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(54) Title: FUSION PROTEINS HAVING INCREASED HALF-LIVES

(57) Abstract

Fusion proteins and DNA molecules encoding such fusion proteins comprising a core protein which is covalently linked to a stabilizing polypeptide wherein the fusion protein is more resistant to proteolytic degradation than the core protein. Methods for making and therapeutic and diagnostic uses for the fusion proteins and DNA molecules encoding the same are also described.

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FUSION PROTEINS HAVING INCREASED HALF-LIVES

FIELD OF THE INVENTION

This invention relates to the production of fusion proteins with increased resistance to proteolytic degradation and their therapeutic and diagnostic uses, including the use of such fusion proteins in the therapeutic treatment of disease.

BACKGROUND OF THE INVENTION

Proteolytic enzymes (proteases) play important roles in the regulation of the activities of other proteins in the cell. For example, proteolytic enzymes are well known to be critical in the formation of active enzymes from inactive proenzymes (zymogens) as found in the stomach or pancreas (for example, chymotrypsinogen, trypsinogen, pepsinogen, procarboxylase, proelastase); in the proteolytic conversions of zymogens to form fibrin to clot blood; in the complement cascade involved in lysing foreign cells and in the inflammatory response; in the synthesis of certain hormones (for example, insulin from proinsulin); in processing proteins for insertion into the cell membrane or secretion from the cell; and in the processing of antigens for presentation by antigen presenting cells of the immune system.

In addition, it has been known for many years that the eukaryotic cell contains at least two distinct systems of enzymes for the continuous degradation of cell proteins (see, for example, Zubay, Biochemistry, pp. 963 - 972 (Addison-Wesley Publishing Co., Reading, Massachusetts, 1983)). The ATP-dependent multisubunit protease (also referred to as the proteosome) is responsible for the degradation of soluble abnormal proteins and soluble, relatively short-lived normal proteins in the cell. This ATP-dependent protease is also involved in the degradation of those proteins to which ubiquitin attaches (ubiquitination) as a signal for degradation (see, for example, Peters, *Trends Biochem. Sci.*, *19*: 377 - 382

(1994)). The other major proteolytic system of eukaryotic cells is the lysosome organelle which is primarily responsible for the degradation of relatively long-lived proteins, membrane proteins, and extracellular proteins. The lysosomal degradative system may also be involved in protein degradation associated with certain pathological conditions, such as muscular dystrophy, denervated muscle conditions, and wounds from burns (Zubay, Biochemistry, 1983). Together, the ATP-dependent protease and the lysosome comprise the primary proteolytic activities responsible for the rate of degradation of most proteins in the cell as reflected in and defined by the half-life of each protein which is the time during which 50 percent of the molecules of a particular protein is degraded.

The half-lives of particular proteins vary widely, for example, ranging from a couple of hours or less for key enzymes of metabolic pathways to days for other proteins. Two models have emerged for predicting whether a protein is likely to have a relatively short or long half-life. In the N-end rule pathway, certain amino acids at the amino-, or N-terminal, end of a particular protein serve as a signal to proteases of the cell and can be used to predict whether a protein will have a relatively short or long half-life (see, Bachmair et al., *Science, 234*: 179 - 186 (1986)). The PEST hypothesis derives its name from the single-letter abbreviations of the four amino acids proline (P), glutamic acid (E), serine (S), and threonine (T). According to this model, proteins containing regions ("PEST domains") rich in one of these four amino acids are likely to have half-lives of 2 hours or less (see, Rogers et al., *Science, 234*: 364 - 368 (1986)).

There is a need in the art for modifying the half-life of a particular protein so as to make it more or less resistant to protease degradation. One object of the invention is to modify a protein so as to provide an increase in its half-life.

SUMMARY OF THE INVENTION

This invention provides the means and methods for producing fusion proteins that are modified to be more resistant to protease degradation. Such fusion proteins comprise the amino acid sequence of a core protein that is normally susceptible to proteolytic degradation and a stabilizing polypeptide described herein. Such fusion proteins are more resistant to degradation by proteases and, thus, have a longer half-life than the unfused core protein.

As used herein, "half-life" refers to the time period over which 50% of the protein molecules are degraded by a protease. Preferably, the half-life of a fusion protein of this invention is at least approximately 10-20% and preferably 25% longer than the corresponding unfused core protein.

Fusion proteins of the invention will include a core protein and a stabilizing polypeptide sequence covalently linked thereto. The stabilizing sequence may be joined to the core protein at the amino (n-) or carboxy (c-) terminus of the core protein or may be inserted within the core protein.

The present invention provides a method for increasing the resistance of a core protein to proteolytic degradation, comprising linking or inserting a stabilizing polypeptide onto or into the core protein, wherein the stabilizing polypeptide has the general formula:

$$[(Gly_a)X(Gly_b)Y(Gly_c)Z]n$$

wherein each Gly_a, Gly_b, Gly_c, independently, may be one, two, three, four, five or six sequential glycine residues; each of X, Y and Z is, independently, selected from the group consisting of alanine, serine, valine, isoleucine, leucine, methionine, phenylalanine, proline, and threonine; wherein X, Y and Z, respectively, need not be identical from n repeat to n repeat;
wherein n is 1 to 66.

The present invention also provides a method for increasing

the resistance of a core protein to proteolytic degradation, comprising linking or inserting a nucleotide sequence encoding a stabilizing polypeptide to a nucleotide sequence encoding a core protein to create a gene fusion which is expressible as a fusion protein, wherein the stabilizing polypeptide has the general formula:

$$[(Gly_a)X(Gly_b)Y(Gly_c)Z]n$$

wherein each Gly_a, Gly_b, Gly_c, independently, may be one, two, three, four, five or six sequential glycine residues; each of X, Y and Z is, independently, selected from the group consisting of alanine, serine, valine, isoleucine, leucine, methionine, phenylalanine, proline, and threonine; wherein X, Y and Z, respectively, need not be identical from n repeat to n repeat;

wherein n is 1 to 66.

The methods of the present invention can be used to increase the resistance of any core protein to proteolytic degradation. Preferably, the core protein retains its biological activity in the fusion protein. The core protein used in the method of the present invention may be any enzyme or other protein such as the herpes virus type 1 Thymidine kinase, Clotting Factors, and insulin.

The invention thus relates to a recombinant protein containing a stabilizing amino acid sequence having the formula:

$$[(Gly_a)X(Gly_b)Y(Gly_c)Z]n$$

wherein each Gly_a, Gly_b, Gly_c, independently, may be one, two, three, four, five, or six sequential glycine residues; each of X, Y and Z is, independently, selected from the group consisting of alanine, serine, valine, isoleucine, leucine, methionine, phenylalanine, proline, and threonine; more preferably, each of X, Y and Z is, independently, selected from the group consisting of alanine, serine, methionine and proline; most preferably, each of X, Y and Z is, independently, alanine and serine; more preferably, each of X and Y is, independently, serine and methionine; more

preferably, each of X and Y is, independently, methionine and proline; more preferably, each of X and Y is not lysine or arginine; wherein each of X, Y and Z, respectively, need not be identical from n repeat to n repeat; and n may be from 1-66.

Preferred stabilizing sequences of the invention include the 235 amino acid sequence provided in Figure 1 and a 17 amino acid glycine-alanine sequence having the amino acid sequence: Gly Ala Gly Ala Gly Ala Gly Gly Ala Gly Ala Gly Gly Gly Ala Gly Gly (see Figure 2).

The invention is particularly useful where administration of a protein is desired, for example, for a therapeutic or diagnostic purpose, where it is desired that the protein has a longer half-life in the recipient mammal, for example a human. According to the invention, a stabilizing sequence is inserted into a foreign protein (also called a core protein) or at either the amino or carboxy terminal end of the foreign protein, thus rendering the resulting chimeric foreign protein (also called the fusion protein) relatively longer-lived upon administration to the recipient. Proteins particularly useful according to the invention will be of therapeutic or diagnostic value to a mammal, such as a human. It is therefore preferred that the protein containing the stabilizing sequence not be a bacterial housekeeping protein such as an RNA polymerase, or beta-lactamase, a kanamycin resistance protein, or certain insect proteins such as silk fibroin proteins such as SLP1, SLP2, SLP3, and SLP4, the EBS1 protein (all as described in WO88/03533).

The invention also features a recombinant fusion protein containing the above-described stabilizing amino acid sequence, wherein the recombinant fusion protein comprises a non-immunogenic core protein and the stabilizing sequence.

Although the stabilizing sequence may be joined to either

the carboxy or amino terminal end of the core protein, or inserted anywhere within the protein to render that protein relatively longer-lived, it is preferred that the stabilizing sequence is inserted within the core protein at a distance wherein either terminal residue of the stabilizing sequence is at a distance of between about 1 and 300 residues from a proteolytic processing site of the protein. Preferably, the core protein contains one or more of such processing sites, the sites being ATP-dependent processing sites. In the case of a core protein containing multiple processing sites, it is believed that all such sites of the core protein will be rendered less susceptible to processing according to the invention.

In its broadest aspect, the stabilizing sequence is present anywhere within or contiguous with the core protein, as the distance between the core protein and the stabilizing sequence is not believed to be critical to increase the half-life of the fusion protein.

However, where any distance is indicated as preferred, the distance between the site of insertion in the core protein of the stabilizing sequence and one amino acid of the core protein proteolytic processing site is preferably between about 1 and 200 residues, more preferably 10-100 residues, and most preferably between about 20 and 50 residues. As used herein, "proteolytic processing site" refers to a stretch of about 8-20 amino acids within a protein that is recognized by a protease. Examples of proteases which target such sites include but are not limited to chymotrypsin-like proteases, such as the multicatalytic cytosolic protease of the proteasome; serine and threonine proteases; and trypsin-like proteases.

The invention also encompasses a recombinant nucleic acid encoding the fusion protein described above, and bacterial or mammalian host cells containing such recombinant nucleic acids.

The invention also encompasses a vector containing a nucleotide sequence encoding the stabilizing sequence, wherein the 5' and 3' ends of this nucleotide sequence have been modified so as to permit insertion of this nucleotide sequence into the coding region of a target protein such that the insertion occurs in the same reading frame as the target protein.

Preferably, the 5' and/or 3' ends of the nucleotide sequence encoding the stabilizing sequence have been modified such that the sequence can be inserted into the coding region of a target core protein in any one of one, two or three upstream selected reading frames (i.e., upstream of the site of insertion in the protein of the stabilizing sequence) and any one of one, two or three corresponding downstream reading frames (i.e., downstream of the site of insertion). Preferably, the 5' and/or 3' ends of the nucleotide sequence encoding the stabilizing sequence have been modified such that the sequence can be inserted into the coding region of a target core protein in-frame.

Preferably, the 5' and/or 3' ends of the nucleotide sequence encoding the stabilizing sequence have been modified to permit joining of this nucleotide sequence to a nucleotide sequence encoding a target core protein such that the nucleotide sequence encoding the stabilizing sequence is joined in-frame at a codon for a selected amino or carboxy terminal-end amino acid of the target core protein.

The invention also encompasses methods of testing for stabilizing sequences which confer on a recombinant fusion protein containing such a sequence fused to a selected core protein an increased half-life, the method comprising the steps of incubating a host cell that expresses a recombinant fusion protein of the invention containing a stabilizing sequence for a time sufficient to permit degradation of the recombinant fusion protein in the host cell, and assaying

the recombinant fusion protein for degradation, for example, by comparing degradation of the recombinant fusion protein and a reference protein, such as the unmodified core protein.

The invention also encompasses methods of testing for stabilizing sequences which confer on a recombinant fusion protein containing such a sequence fused to a selected core protein an increased half-life, the method comprising the steps of incubating the recombinant fusion protein of the invention containing a stabilizing sequence with a protease in vitro for a time sufficient to permit degradation of the recombinant fusion protein, and assaying the recombinant fusion protein for degradation, for example, by comparing degradation of the recombinant fusion protein and a reference protein, such as the unmodified core protein.

The invention also encompasses methods of testing for stabilizing sequences which confer on a recombinant fusion protein containing such a sequence fused to a selected core protein an increased half-life, the method comprising the steps of providing an animal which contains the recombinant fusion protein, and assaying the half-life of the recombinant fusion protein as obtained from a tissue or fluid of the animal over a time period sufficient to observe degradation of the fusion protein. The animal may contain a recombinant DNA encoding and expressing the recombinant fusion protein or the recombinant fusion protein may be administered to the animal and assayed over time.

The invention also encompasses methods of treating a disease in a mammal, comprising administering to a mammal nucleic acid encoding a recombinant fusion protein comprising the stabilizing sequence inserted in frame with a nucleic acid molecule encoding a selected core protein as described herein, which core protein has a shorter half-life in the absence of the stabilizing sequence, i.e., as an unfused core protein, relative to a longer half-life in the presence

of such sequence, i.e, as part of a fusion protein described herein.

The invention also encompasses the use of the recombinant fusion protein or nucleic acid encoding the recombinant fusion protein in the manufacture of a medicament for the treatment of a disease in a mammal.

The invention also encompasses the recombinant fusion protein or nucleic acid encoding the recombinant fusion protein for use in therapy.

The invention provides fusion proteins which comprise a non-immunogenic core protein covalently linked to a stabilizing polypeptide, the non-immunogenic core protein being any protein which retains its biological activity in the fusion protein form and which does not lead to an immunogenic reaction in a mammalian host in its unmodified form.

In a preferred embodiment, this invention provides fusion proteins which comprise an IκB regulator protein covalently linked to a stabilizing polypeptide, and genes encoding such proteins. Such fusion proteins have a longer half-life, that is, at least approximately 10-20% and preferably 25% longer than the unfused IκB regulator, and retains the biological activity of the native protein, and are useful in treating inflammatory bowel disease (IBD).

In a preferred embodiment, this invention provides fusion proteins which comprise a nitroreductase protein covalently linked to a stabilizing polypeptide which retains the biological activity of the native protein, and genes encoding such proteins. Such fusion proteins are useful to activate nitro drugs such as CB1954, and thus are useful in enzyme/prodrug therapy to treat cancer or other pathological conditions.

In another embodiment of this invention, DNA molecules encoding fusion proteins comprising an IκB regulator protein or nitroreductase linked to a stabilizing polypeptide are provided for use in gene therapy, the IκB fusion protein to treat IBD and the nitroreductase fusion protein to treat cancer or a pathological disease.

Owing to their longer half-lives, fusion proteins of this invention also are useful as improved reagents in diagnostic methods, including in vivo imaging techniques such as MRI.

Further features and advantages of the invention will become more fully apparent in the following description of the embodiments and drawings thereof and from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequence of the 235 residue glycine/alanine repeat region from Epstein Barr Virus EBNA1 protein.

Figure 2 shows a nucleotide sequence and encoded 17 amino acid stabilizing polypeptide.

Figure 3 shows the nucleotide sequence of the coding strand of DNA and deduced amino acid sequence for a 266 amino acid stabilizing polypeptide and is the FLGA insert described herein. Each amino acid of the amino acid sequence is depicted by its upper case, single letter abbreviation.

Figure 4A shows a schematic pBSFLGA1 vector containing Gly-sequence coding region for protein engineering. The plasmid is based on the pBS KS (+) cloning vector.

Figure 4B shows the nucleotide sequence of the stabilizing sequence-encoding insert with 5' and 3' flanking regions.

Figure 5 is a schematic map of the pBSFLGA2 vector containing Gly-sequence coding region for protein engineering. The plasmid was created from pBSFLGA1 by

filling the protruding BamHI ends with Klenow enzyme and ligation. The BamHI site is substituted by an artificial ClaI site.

Figure 6 presents three upstream frames and 3 downstream frames which can be obtained using different combinations of restriction enzymes and/or Klenow enzyme and mung bean nuclease.

Figure 7 presents FLGA containing constructs with translation start (pBS-M-FLGA) or stop codons (p6hisMetFLGAstop) for expression of N-terminal and C-terminal FLGA fusion proteins.

Figure 8 is a diagram illustrating the cloning of a recombinant DNA encoding the fusion protein IkB-N-flGA.

Figure 9 is a diagram of maps of chimeric IκB genes.

Figure 10 is a diagram of the cloning of HA-tagged IkB chimeric genes.

Figure 11 is an autoradiogram of polyacrylamide gels showing in vitro ATP-dependent degradation of IκB and IκB fusion proteins.

Figure 12 is an autoradiogram of a polyacrylamide gel showing in vivo degradation of IκB and IκB fusion proteins.

Figure 13 shows the amino acid sequence for murine IκB-α.

Figure 14 shows a nucleotide sequence encoding murine IκB-α. The translational start codon (atg) and translation stop codon (taa) of the IκB-α structural coding sequence are underlined.

Figure 15 shows the amino acid sequence for human IκB-α.

Figure 16 shows a nucleotide sequence encoding human IκB-α. The translational start codon (atg) and translational stop codon (tga) of the IκB-α structural coding sequence are underlined.

Figure 17 is a schematic illustration of construction of a vector (pJG-N-NTR) containing a gene encoding a fusion protein according to the invention in which the stabilizing polypeptide is attached to the amino terminus of the fusion protein..

Figure 18 is a schematic illustration of construction of a vector containing a gene encoding a fusion protein in which

the stabilizing polypeptide is attached at the carboxy terminal end of the protein

Figure 19 shows the predicted amino acid sequence of the junction region of two of the Gly-Ala minimal repeat constructs with 25 and 33 glycine/alanine residues, respectively, in the stabilizing polypeptide.

Figure 20 shows the predicted amino acid sequence of the junction region of two of the Pro-Ala minimal repeat constructs with 17 and 25 proline/alanine residues, respectively, in the stabilizing polypeptide.

Figure 21 (A) is a schematic representation of the chimeric IkBα constructs. The coding sequence of the IkBα is shown as an open box with the five ankyrin repeats indicated by hatched boxes and the encoding region for three influenza hemagglutinin epitopes (3HA) indicated by a filled box.

Figure 21 (B) presents the amino acid sequences of the glycine/alanine inserts of Figure 21 A.

Figure 22 (A) is a western blot of total cell extracts probed with an IkBα-specific rabbit serum. Ectopically expressed IkB recombinant proteins are indicated by filled arrows. Open arrows indicate the endogenous IkBα which is recognized by the specific rabbit serum and serves as internal control for degradation.

Figure 22 (B) represents the ratio between the intensity of the IkB specific bands (as determined by scanning of the ECL developed blots) before treatment and after treatment with TNFα for the indicated time.

Figure 23 shows signal dependent degradation of IkB chimeras containing a 24 amino acid long Gly-Ala repeat.

Figure 24 shows results of Western blots of immunoprecipitation experiments in which IkB proteins containing the Gly-Ala repeat are bound to NFkB/RelA heterodimers.

Figure 25 (A) is a Western blot of HeLa cells transfected with GA24-X and 3HA-IkB and treated with 100 ng/ml of recombinant TNFα with or without addition of 10 nM of the peptide aldehide MG123 and LLnL to inhibit proteasome activity, and the ectopic IkB was then immunoprecipitated

from ocadaic acid containing extracts using the anti-3HA mAbs, and the blots probed with the anti-IkB serum.

Figure 25 (B) is a Western blot showing ubiquitination of 3HA-IkB and GA24-X in TNFα treated HeLa cells in the presence of the peptide aldehyde MG123 and LLnL. IkB was imrnunoprecipitated from cell extracts containing 20 mM of the isopeptidase inhibitor NEM using the anti IkB rabbit serum and blots probed with the anti-HA antibody.

Figure 25 (C) is a Western blot in which the high molecular weight IkB contains ubiquitin conjugates. Transfected HeLa cells were treated as described above and total cell extracts were immunoprecipitated with the anti-HA mAb, and the blot probed with an anti-ubiquitin serum.

Figure 26 is a Western blot showing that a ubiquitinated chimera containing Gly-Ala repeats is not targeted to proteasomes.

Figure 27 (A) shows results of Molecular Dynamics Phosphorimager image of SDS-PAGE. The time of chase (min) is indicated at the top. The arrows mark the bands, 3HA-IkB and GA24-X, respectively, that have been chosen for quantitative analysis. Transfected HeLa cells were metabolically labelled in the presence of [^{35}S]Met, and then incubated in isotope free medium for the indicated time before immunoprecipitation with anti-3HA antibodies.

Figure 27 (B) shows densitometry analysis in which % residual protein was calculated as the ratio between the intensity of the specific band at the indicated time of chase and the intensity of the band at time 0.

Figure 28 (A) shows results of apoptosis determination in which HeLa cells were cotransfected with the indicated IkB construct and with a β-gal plasmid. After 48 hrs the transfected cells were treated with 10 ng/ml TNPα for 18 hrs and apoptotic cells were then detected by Hoechst staining.

Figure 28 (B) shows representative Hoechst staining as described in Figure 28(A).

Figure 29 (A) shows Western blot analysis of total cell lysates probed with the anti hemagglutining tag antibody 12CA5. Time kinetics of TNFA induced degradation are shown

for IkB chimeras containing a 24 amino acid long Gly-Ala repeat (GA24-N) or a polyglycine sequence of similar length (GG24-N IkB).

Figure 29 (B) shows results of densitometry analysis of Figure 29 (A).

Figure 30 presents graphs showing sensitivity of EBNA4 chimeras to ubiquitin/proteasome dependent degradation.

DESCRIPTION

This invention is based on the discovery that linking certain stabilizing polypeptides to a protein of interest (the core protein), will produce a fusion protein which retains the biological activity of the core protein and which is more resistant to protease degradation (or processing) than the unmodified core protein. The fusion proteins produced by the method of the invention are thus more stable, as evidenced by an increased half-life, and are able to exert their activity or function for a longer period of time than the corresponding unmodified core proteins. A particular application of this invention is the use of the fusion proteins of the present invention to treat autoimmune disease such as inflammatory bowel disease (IBD).

Stabilizing Polypeptides and Fusion Proteins

When a stabilizing polypeptide of this invention is linked to a protein of interest, the resulting fusion protein is more stable and resistant to proteolytic degradation than the unfused protein (referred to as the core protein). A fusion protein produced by the method of the present invention is able to evade proteolytic degradation, has an increased half-life, and, thus, is able to exert its activity or function in the cell for a longer period of time than the corresponding unfused core protein. Such beneficial or therapeutic activities include, but are not limited to, the control over expression of other proteins that contribute to or cause disease, as in autoimmune disease, cancer, and abnormal inflammation.

Preferably, the fusion proteins produced by the method of the present invention are at least about 10-20% more resistant to protease degradation than the unmodified core protein of interest.

A stabilizing amino acid sequence for insertion or linkage to a core protein according to the invention will fall within the general formula:

$[(Gly_a)X(Gly_b)Y(Gly_c)Z]n$

wherein each Gly_a, Gly_b, Gly_c, independently, may be one, two, three, four, five, or six sequential glycine residues; each of X, Y and Z is, independently, selected from the group consisting of Ala, Ser, Val, Ile, Leu, Met, Phe, Pro, and Thr; more preferably, each of X, Y and Z is, independently, selected from the group consisting of Ala, Ser, Met and Pro; most preferably, each of X, Y and Z is, independently, Ala and Ser; more preferably, each of X and Y is, independently, Ser and Met; more preferably, each of X and Y is, independently, Met and Pro; more preferably, each of X and Y is not Lys or Arg; wherein each of X, Y and Z, respectively, need not be identical from n repeat to n repeat; and n may be from 1-66.

In other preferred embodiments, the stabilizing sequence comprises (GlyGlyXGlyYGlyZ) and n=7 repeats; or (GlyGlyXGlyYGlyGlyZ) in n=9 repeats where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total repeat number of 66, and preferably 28 total repeats; or the stabilizing sequence is (GlyGlyXGlyYGlyGlyGlyZ) in n=7 repeats where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total repeat number of 66, and preferably 28 total repeats; or the stabilizing sequence comprises (GlyGlyAlaGlyAlaGlyGlyAla) in n=9 repeats where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total repeat number of 66, and preferably 28 total repeats.

Most preferably, the stabilizing sequence is (GlyGlyAlaGlyAlaGlyGlyGlyAla) in n=7 repeats where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total repeat number of 66, and preferably 28 total repeats; or the stabilizing sequence is (GlyGlyXGlyYGlyGlyZ) in n=9 repeats where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total n=66; and (GlyGlyXGlyYGlyGlyZ) in n=9 repeats where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total n=28.

The stabilizing sequence (GlyGlyAlaGlyAlaGlyGlyAla) in n=9 repeats and (GlyGlyAlaGlyAlaGlyGlyGlyAla) in n=7 repeats is also preferred, where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total repeat number of 66.

In other preferred embodiments, the stabilizing sequence comprises (GlyGlyXGlyYGlyZ) and n=7 repeats; or (GlyGlyXGlyYGlyGlyZ) in n=9 repeats where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total repeat number of 66, and preferably 28 total repeats; or the stabilizing sequence is (GlyGlyXGlyYGlyGlyGlyZ) in n=7 repeats where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total repeat number of 66, and preferably 28 total repeats; or the stabilizing sequence comprises (GlyGlyAlaGlyAlaGlyGlyAla) in n=9 repeats where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total repeat number of 66, and preferably 28 total repeats.

Most preferably, the stabilizing sequence is (GlyGlyAlaGlyAlaGlyGlyGlyAla) in n=7 repeats where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total repeat number of 66, and preferably 28 total repeats; or the stabilizing sequence is (GlyGlyXGlyYGlyGlyZ) in n=9 repeats where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total n=66; and (GlyGlyXGlyYGlyGlyZ) in n=9 repeats where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total n=28.

The stabilizing sequence (GlyGlyAlaGlyAlaGlyGlyAla) in n=9 repeats and (GlyGlyAlaGlyAlaGlyGlyGlyAla) in n=7 repeats is also preferred, where the remaining n repeats comprise $(Gly_a)X(Gly_b)Y(Gly_c)Z$ up to a total repeat number of 66.

Recombinant (i.e., made by recombinant DNA techniques) isolated peptides of the invention will not include synthetic (i.e., chemically synthesized) polypeptides which are used in the prior art for generation of antibodies to Epstein Barr Virus, for example, the synthetic peptides described in WO90/01495:

P60(C): G G G A G A G G A G A G G G GA; P27(A): K G T H G G T G A G A G A G G A G A G; P62(B): A G A G G G A G G A G A G G G A G G A G and peptides thereof G G G A G G A G A G G G A G G A G, A G G A G A G G G A G G A G, A G A G G G A G G A G; F14: G G A G G A G G A G A G G G A G; F15: G A G G A G A G G G A G G A G G; F16(G): G G A G A G G A G A G G A G A G A G G A G A G, although such peptides, or certain stabilizing portions thereof, may of course be useful in the method of the present invention to prepare fusion protein.

A preferred stabilizing sequence is the 235 amino acid glycine repeat sequence of Figure 1.

The nucleotide sequence of the coding strand of DNA and deduced amino acid sequence for a 17 amino acid stabilizing polypeptide useful according to the invention is shown in Figure 2.

In numerous experiments described herein, chimeric constructs bearing a stabilizing sequence of 24 amino acids in length (GA24 constructs) have been assayed; however, stabilizing sequences of shorter chain lengths are also of use according to the invention. Using the cloning protocol by which GA24-X or GA24-N were cloned, the following putative stabilizing sequences are incorporated into a fusion protein construct (wherein G = glycine and A =

alanine);

NH_2-GAGA-COOH

NH_2-GGAG-COOH

NH_2-GGAGAGAG-COOH

NH_2-GGAGGAGAGGAGAGAG-COOH

A fusion protein construct containing a putative stabilizing sequence is employed according to the methods described herein by which the GA24 constructs are tested for inhibition of degradation by proteases.

A stabilizing polypeptide described herein, as well as fusion proteins comprising such a polypeptide linked to a core protein, may be prepared using standard synthetic polypeptide methods or by recombinant nucleic acid methods known in the art (see examples, below). Such fusion proteins exhibit a half-life that is at least approximately 10% longer than the corresponding unmodified core protein, as determined using a standard protease degradation assay available in the art. The "half-life" of a particular protein refers to the time period during which one-half of the protein molecules of that particular protein in the cell or a mixture are degraded.

The half-life of a polypeptide or protein of this invention can be determined using <u>in vitro</u> or <u>in vivo</u> methods. For example, a fusion protein of this invention can be incubated in the presence of a cell-free rabbit reticulocyte lysate containing the components of the ATP-dependent, proteosome and ubiquitin cascade system. The percent protein degraded over time can be assessed by either separation of the proteins on SDS-PAGE followed by densitometry or analysis of trichloroacetic acid (TCA) precipitation of proteins. The specific degradation of proteins in this particular cell-free system are measured after subtraction of background degradation in the absence of ATP.

The half-life of polypeptides and proteins produced by the

method of the present invention may also be determined in an in vivo system by measuring degradation of recombinant proteins expressed in cells which express or are induced to express a protease. For example, if an antibody to the expressed recombinant protein is available, the degradation of recombinant protein expressed in the cell can be followed using SDS-PAGE and standard Western blot analysis.

A more complete appreciation of this invention and the advantages thereof can be obtained from the following non-limiting examples.

The present invention also provides fusion proteins comprising a core protein and a stabilizing polypeptide wherein the core protein in its unmodified form is non-immunogenic.

The present invention also provides recombinant nucleic acid encoding the fusion protein of the present invention.

In a specific embodiment of the invention, there are provided fusion proteins which comprise a prodrug activating enzyme, nitroreductase, and a stabilizing polypeptide, and DNAs encoding such proteins.

In another specific embodiment of the invention, there are provided fusion proteins which comprise an IκB regulator protein and a stabilizing polypeptide and DNA molecules encoding such fusion proteins. Such fusion proteins and the DNA molecules encoding such proteins are useful in the treatment of inflammatory bowel disease.

Inflammatory bowel disease (IBD) in humans is a chronic inflammation of the gastrointestinal tract characterized by a variety of symptoms including severe diarrhoea, weight loss, intestinal lesions, and rectal prolapse. At least two distinct forms of IBD are recognized clinically: ulcerative colitis, which is a predominantly superficial, ulcerative

inflammation of the large intestine, and Crohn's disease, which is characterized as a transmural, granulomatous inflammation that may occur anywhere in the gastrointestinal tract. The pathogenesis of IBD is correlated with an abnormal elevation in the level of expression in and secretion from lymphocytes and macrophages of cytokines that promote inflammation (the proinflammatory cytokines). Thus, lamina propria macrophages isolated from patients with Crohn's disease produce and secrete into culture medium relatively high levels of the proinflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (see, for example, Neurath et al., *Nature Medicine, 2*: 998 - 1004 (1996)). Animal models of IBD also have been shown to contain lymphocytes or macrophages in areas of inflammation which produce elevated levels of proinflammatory cytokines (Neurath et al., 1996).

Like many genes whose products are involved in the immune response or inflammation, the transcription of genes encoding proinflammatory cytokines is regulated by transcription factors of the NF-κB family of proteins. Members of NF-κB family include NF-κB-1 (also known as p50 and its larger precursor, p105), p65 (also known as RelA), NF-κB-2 (also known as p52 and its larger precursor, p100), c-Rel (also known simply as Rel), and RelB. NF-κB transcription factors are known to bind a specific 10 base pair sequence, which has a consensus sequence of GGGACTTTCC present in the promoter/enhancer region of those cytokine genes under NF-κB control.

The activity of the NF-κB transcription factors themselves is regulated in normal lymphocytes and macrophages. For example, p65 and p50 can associate with one another as subunits of an active NF-κB transcription factor to stimulate transcription of various genes, including genes encoding proinflammatory cytokines. However, in normal cells, the IκB-α regulator protein binds both p65 and p50 to prevent the formation of an active NF-κB transcription

factor and, thereby, prevent overproduction of proinflammatory cytokines and attendant onset of IBD (Baeuerle et al., *Science, 242*: 540 - 545 (1988); Thompson et al., *Cell, 80*: 573 - 582 (1995). When the IκB-α regulator protein is eventually degraded, the p65 and p50 subunits become free to associate and again form an active NF-κB transcription factor. Thus, the availability of adequate intracellular levels of intact (non-degraded) IκB-α regulator protein to bind the p65 and p50 subunits is critical to maintaining a control over the inflammation process in normal tissue. If an abnormality exists that results in the expression of unusually high levels of one or both of the p65 and p50 subunits, as in Crohn's disease, the formation of active NF-κB transcription factor may overwhelm the capacity of the normal levels of IκB regulator protein to bind p65 and p50 subunits and prevent the onset of IBD. This model of regulation of expression of proinflammatory cytokines is supported by the finding that immunosuppressive corticosteroids used to treat IBD can induce transcription of the IκB-α gene and also by the finding that p65 antisense oligonucleotides abrogate IBD in animal models (Neurath et al., 1996).

Studies of the IκB-α regulator protein have shown that this protein has a half-life of 1 - 2 hours when complexed with NF-κB and is less stable when free in the cytoplasm (see, for example, Rice et al., *EMBO J., 12*: 4685 - 4695 (1993); Scott et al., *Genes & Dev., 7*: 1266 - 1276 (1993); Sun et al., *Science, 259*: 1912 - 1915 (1993)). Beauparlant et al. (*J. Biol. Chem., 271*: 10690 - 10696 (1996)) have suggested that the relatively short half-life of the IκB-α regulator protein is likely to be due to the fact that a carboxy terminal region of the protein is rich in proline, glutamic acid, serine and threonine amino acids, consistent with the PEST hypothesis (Rogers et al., *Science, 234*: 364 - 368 (1986)). A variety of studies have demonstrated the importance of various amino acid residues of the amino terminal region of IκB-α in phosphorylation, ubiquitination,

and degradation by the multicatalytic protease (proteosome) (see, for example, Chen et al., *Genes & Dev., 9*: 1586 - 1597 (1995); Traenckner et al., *EMBO J., 13*: 5433 - 5441 (1994)). Various residues of the carboxy terminal region of IκB-α are critical to preventing degradation (see, for example, Brockman et al., *Mol. Cell. Biol., 15*: 2809 - 2818 (1995); Brown et al., *Science, 267*: 1485 - 1488 (1995); Whiteside et al., *Mol. Cell. Biol.,15*: 5339 - 5345 (1995)). Beauparlant et al. (*J. Biol. Chem., 271*: 10690 - 10696 (1996)) demonstrated that deletions from amino acids 269 to 287 of IκB-α abolish inducer-mediated degradation by rendering IκB-α constitutively unstable and diminish the association of IκB-α with p65.

The nucleotide coding sequence and corresponding amino acid sequences for the IκB-α protein from a number of species are known, including murine IκB-α (see, GenBank accession number MMU36277, Figures 13 and 14) and human IκB-α (see, GenBank accession number M69043, Figures 15 and 16). Such sequence data permit the manipulation and use of IκB-α protein in a variety of molecular studies. For example, Lin et al. (*Mol. Cell. Biol., 16*: 2248 - 2254 (1996)) showed that the protein p105, the precursor to the p50 subunit of NF-κB, contains a glycine-rich region (GRR) which includes the 23 amino acid sequence Gly Gly Gly Ser Gly Ala Gly Ala Gly Gly Gly Gly Met Phe Gly Ser Gly Gly Gly Gly Gly Ser Thr and which appears to act as a processing signal for the precursor p105 by directing an endoproteolytic cleavage downstream of the GRR. In one example, Lin et al. tested the GRR in a fusion protein containing an amino terminal IκB-α protein sequence linked to the GRR which in turn was linked to a p50 protein sequence as the carboxy terminal region of the fusion protein. The fusion protein was processed in COS1 cells to form both an IκB-α-like product (the IκB-α protein sequence with the GRR and some sequences from p50) and a p50 peptide cleavage product (Lin et al., 1996).

The critical role played by the IκB regulator protein in the

normal inflammatory process and the availability of nucleotide and amino acid sequences make this protein a particularly attractive target for modification to increase the protein's half-life and, thereby, increase its effectiveness in controlling the formation of active NF-κB and the onset of inflammatory bowel disease.

RECOMBINANT FUSION PROTEINS CONTAINING STABILIZING POLYPEPTIDES AND GENE FUSIONS ENCODING SUCH FUSION PROTEINS

1. Construction of Gene Fusions Encoding Fusion Proteins Containing Stabilizing Polypeptides.

Recombinant gene fusions of this invention are prepared by using standard recombinant DNA techniques to insert a first DNA molecule encoding a stabilizing polypeptide described herein into the same reading frame of a second other DNA molecule encoding a core protein whose resistance to proteolytic degradation, i.e., its half-life in the presence of a protease, is to be enhanced. The first DNA molecule encoding the stabilizing polypeptide may be attached in-frame at the 5' or the 3' end of the second other DNA molecule encoding the core protein to form a recombinant gene fusion encoding a fusion protein that comprises the amino acid sequences and functions of both the protease inhibitor polypeptide and the core protein. In another embodiment of this invention, the DNA molecule encoding the stabilizing polypeptide may be inserted in-frame within the structural coding sequence of a DNA molecule encoding a core protein to yield a recombinant gene fusion that encodes a fusion protein that comprises the amino acid sequence of the stabilizing polypeptide and a portion of the core protein structure to preserve its original function or activity.

In a preferred embodiment of this invention, a stabilizing polypeptide is covalently linked to the nitroreductase core protein to produce a fusion protein. This fusion protein is especially useful in treating diseases involving depletion of a selected cell population,

such as in activated prodrug treatment of cancer cells.

In another preferred embodiment of this invention, a stabilizing polypeptide is covalently linked to the IκB-α regulator protein (core protein) to produce a fusion protein which is more resistant to protease degradation so that the fusion protein exhibits a longer half-life than the unmodified IκB-α core protein and is able to regulate (inhibit) formation of active NF-κB transcription factor. Such fusion proteins inhibit production of proinflammatory cytokines and are useful for treating IBD. As mentioned above, the nucleotide coding sequence and corresponding amino acid sequence for both murine and human IκB-α regulator proteins are known (see, GenBank accession number MMU36277, Figs. 13 and 14 for murine IκB-α; see, GenBank accession number M69043, Figs. 15 and 16 for human IκB-α) permitting the preparation of fusion proteins using synthetic methods as well as recombinant DNA methods as described in the examples below.

2. Cassettes and Plasmids for Insertion of a Stabilizing Polypeptide into a Core Protein of Interest

In order to obtain a recombinant fusion protein according to the invention, a vector containing a nucleotide sequence encoding a core protein or polypolypeptide is prepared. The core protein is then rendered resistant to protease degradation by inserting in-frame into the core protein coding sequence a nucleotide sequence encoding a stabilizing polypeptide of this invention. The stabilizing polypeptide-containing sequence may be inserted 5' or 3' to, or even within, the coding sequence of the core protein in the same reading frame as long as the resulting fusion protein retains the activity of unmodified core protein.

A recombinant gene fusion of this invention can be made using a DNA cassette which is a DNA molecule which encodes a stabilizing polypeptide of this invention and which is

designed so that it can be inserted at a selected site in another DNA molecule or gene encoding a core protein. DNA cassettes may be designed and synthesized using standard DNA synthetic methods or by ligating synthetic DNA linker molecules to a cloned DNA molecule. DNA cassettes may also be designed to encode a stabilizing polypeptide when inserted into more than one possible reading frame, thereby facilitating in-frame gene fusion with the gene or DNA molecule encoding a core protein.

An example of a DNA cassette used to insert sequence encoding a stabilizing polypeptide into a DNA sequence encoding a target protein was prepared as follows.

A full length stabilizing polypeptide coding region was excised from the EBNA1 gene on plasmid pBSEBNA1 using AvaII and MspI restrictases. The ends were filled in by Klenow enzyme and the blunt-ended fragment was cloned into the pBS KS (+) vector previously linearized by SmaI. The orientation of the insert and was estimated by dideoxysequencing from two directions, using both, universal, and reverse sequencing primers. The resulting plasmid pBSFLGA1 containing a full length EBNA1/glycine-rich polypeptide sequence is shown on Figure 4A. The nucleotide sequence of the insert encoding the stabilizing sequence with 5 and 3 flanking regions is shown in Figure 4B. Figure 3 shows the nucleotide sequence of the coding strand of DNA and deduced amino acid sequence for a 266 amino acid stabilizing polypeptide and is the FLGA insert.

The derivative plasmid pBSFLGA2 contains an additional ClaI site upstream of the 5' end of the stabilizing polypeptide coding region and lacks the BamHI site in the same position (see, Figure 5). This plasmid was created by digesting pBSFLGA1 with BamHI, filling the cohesive termini by Klenow enzyme, and ligating the blunt termini. The constructs allow cloning of the 235 amino acid stabilizing EBNA-1 polypeptide coding region into different sites of a target

gene encoding a core protein of interest. Six possible reading frames can be obtained as is shown in Figure 6. The 244 amino acid sequence referred to as "GA REPEAT 244 AA" in Figure 6 includes the 235 amino acids of the EBNA-1 stabilizing polypeptide of EBNA-1 of Figure 1 and an additional 9 codons which are created by the polylinker cloning sequences on each end of the insert.

3. Tagging recombinant fusion proteins

Plasmid pBS-6xHis-FLGA-stop (Figure 7) encodes a histidine tag, i.e., a sequence of six histidine codons, at the amino terminus of the prospective fusion protein. The target core protein gene or DNA molecule can be inserted into the BamHI site of the vector resulting in an in-frame fusion of the DNA sequence coding for the stabilizing polypeptide at the carboxy terminus of the coding sequence of the core protein DNA. Thus, the expressed fusion protein has an amino terminal histidine tag (6xHis) linked to the core protein sequence linked to the stabilizing polypeptide. Alternatively, the EcoRI-HindIII multiple cloning site can be used to encode a fusion protein having an amino terminal histidine tag polypeptide linked to the stabilizing polypeptide linked to a carboxy terminal core protein sequence. The 6xHis tag can be used for the rapid checking of reading frames by expression in E. coli and for rapid specific purification on Ni beads. After checking for proper reading frame, the resulting gene fusion can be recloned in any eukaryotic vector.

The plasmid pBSMetFLGA (Figure 8) contains a methionine codon immediately upstream of the stabilizing polypeptide sequence and can be used for preparing a fusion protein which has a stabilizing polypeptide linked to the amino terminus of a core protein by inserting the gene or DNA molecule encoding the core protein into the EcoRI site.

The above-described plasmid is useful for providing a DNA

fragment encoding a stabilizing polypeptide sequence such that the DNA fragment can be inserted in-frame into a selected gene encoding a core protein of interest. A DNA sequence encoding any stabilizing polypeptide as defined herein may be modified as described above such that the sequence is insertable in-frame into a selected protein-coding sequence. For example, the nucleotide sequence and corresponding amino acid sequence for a 17 amino acid stabilizing polypeptide (see Figure 2) may be used in place of the 235 amino acid EBNA-1 stabilizing polypeptide (Figure 1), or the 266 amino acid polypeptide (Figure 3) described in the cassettes and plasmids.

Alternatively, where another stabilizing sequence is selected according to the invention and guidance for such sequence selection provided herein, it may be made according to any cloning strategy known to one of skill in the art. Two alternative strategies are described below; i.e., by PCR amplification of a prototype sequence or by oligomerization of a selected minimal motif. The advantage of this latter strategy is that it can easily be modified to give repetitive sequences where the intervening X or Y amino acid or the Glycine backbone can be mutated in different combinations.

1. PCR Amplification

Where a DNA fragment encoding a stabilizing sequence is available, the fragment may be used for PCR amplification, as follows.

A 1122bp long XmaI fragment of the prototype B95.8 BamHIK region containing the Glycine repeat sequence (coordinates 108117-109239 as described in Baer et al., Nature 310:207-211, 1984) may be used as template for PCR amplification. PCR primers are chosen immediately upstream and downstream of the repeats. Artificial BamHI and EcoRI sites are included in the sequences of the 5' and 3' oligonucleotides

respectively. Amplification under low stringency conditions and the repetitive nature of the template allows for multiple priming and generation of PCR products of variable size. For example, the primer pair 5'-AAGGATCCAAGTTGCATTGGATGCAA-3' and 5'-TGAATTCTCGACCCCGGCCTCCACTG-3' may be used in a PCR reaction as follows: 50nM of each primer ad 10-20 ng of each template are mixed in a 50 μl reaction buffer containing 1mM Tris-HCl pH7.5, 5mM KCl, 0.15-0.5 nM $MgCl_2$, 0.001% gelatin, 200 μM dNTP and 2 units Taq DNA polymerase. Amplification conditions include 30-35 cycles of denaturation at 95° C for 1 min, annealing at 45-60° C for 1 min, and elongation at 72° C for 1 min.

The PCR products are cloned in the BamHI and EcoRI sites of the pGEX-T2 vector downstream of the glutathione S-transferase gene (GST). The fusion protein GST-GlyAla is expressed in bacteria using the tac promoter. Expression of fusion proteins containing the repeats inserted in-frame downstream of the GST gene is then screened by Western blotting of lysates from single transformed colonies using affinity purified human antibodies specific for the EBNA1 GlyAla repeat (Dillner et al., 1984, Proc. Nat. Aca. Sci. 81;4652). Screening is performed after induction of individual bacterial clones grown in microwell plates with 0.3 mM IPTG in 150 μl LB medium for 4 hr. 80 μl of bacterial cell suspension is mixed with an equal volume of SDS-PAGE loading buffer and dotted onto nitrocellulose filters using a dot-blot apparatus. The filters are processed according to standard Western blot procedures and developed by ECL (Amersham).

Colonies expressing the GST-GlyAla polypeptide are further characterized to determine the size and coding capacity of the insert by restriction endonuclease analysis and sequencing.

2. Oligomerization of a selected minimal motif.

A set of complementary oligonucleotides encoding a core stabilizing motif are synthesized with 5' and 3' overhangs to allow for oligomerization. This strategy offers the advantage of producing known sequences that can easily be modified to include alternative amino acids.

Examples of complementary oligonucleotides encoding the core motif GlyAlaGlyAlaGlyGlyAlaGly and modifications thereof are provided in Table 1.

The expected coding capacity upon insertion in positive or negative orientation relative to the direction of transcription is presented in Table 2.

After annealing to form a duplex, the oligonucleotides will contain 5' and 3' overhangs corresponding to the initial amino acid codon and the first base of the adjacent codon to allow the formation of head-to-tail multimers upon ligation. Annealing is performed in a 50μl reaction containing 100 μM of each primer, 0.1 M $MgCl_2$, 10 mM Tris-HCl pH7.4. The reaction mix is heated at 72°C for 5 min and allowed to proceed at 65°C for additional 40 min. Ligation is performed by adjusting the annealing mix to 50 mM Tris pH 7.4 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermidine, 1 mM ATP, 100 ng/ml BSA and by adding 10 u of T4 DNA ligase. The reactions are run for 1, 3, 6, 9 and 12 hrs at 15°C. Filling-in of the 3' recessed ends is performed with 0.1 u of the Klenow fragment of DNA polymerase in 50 mM Tris-HCl pH7.5, 7mM $MgCl_2$, 1 mM DTT and 20 μM dNTPs for 20 min at room temperature. Linear multimeric molecules are blunt-end ligated into the SmaI site of the pGEX-T2 vector as shown in Table 3.

Clones expressing the GST-repeat fusion protein are selected by reactivity with GlyAla specific antibodies, as described herein. For fusion proteins containing repeats for which specific antibodies are not available, selection is performed on the basis of size after purification from the

bacterial lysates on GST-binding glutathione-coated sepharose beads. Expressing clones are selected for further characterization of the inserts.

Plasmids are digested with BamHI and EcoRI that cut immediately upstream and downstream of the SmaI site in the pGEX-T2 vector. The inserts are size fractionated in 3% agarose gels and visualized by UV irradiation after ethidium bromide staining, Alternatively, plasmids and insert fragments obtained after BamHI/EcoRI digestion are end labelled with 1 unit Klenow fragment of DNA polymerase per μg plasmid in 25 μl reactions containing 50 mM Tris-HCl pH 7.5, 10 mM MgSO4, 1 mM DTT, 50μg/ml BSA Pentax fraction V, 2 nmoles dNTPs and 2pmoles α-^{32}P-dCTP. Size fractionation is performed in 8% acrylamide gels.

A stabilizing sequence obtained according to the procedures described above is subcloned into a polylinker of an appropriate vector to generate subcloning cassettes containing unique upstream and downstream restriction sites. For example, subcloning into the BamHI and EcoRI sites of the pBluescript-SK vector (Stratagene) will generate subcloning cassettes containing unique upstream sites: SacI, BstXI, NotI, XbaI, and SpeI, and unique downstream sites HindIII, ClaI, HincII, AccI, SalI, XhoI, ApaI, DraII and KpnI.

TESTING OF FUSION PROTEIN CONTAINING STABILIZING POLYPEPTIDE FOR RESISTANCE TO PROTEIN DEGRADATION

The ability of a stabilizing polypeptide, as defined herein, when linked to a core protein of interest, to inhibit ubiquitin and proteasome-dependent degradation of the core protein, is tested in <u>in vitro</u> and/or <u>in vivo</u> protein degradation assays. For example, such assays can be used to test the degradation of intact full-length IκB core protein and a fusion protein consisting of the IκB amino acid sequence linked to a stabilizing polypeptide. These

experiments are designed to show that a fusion protein containing a glycine and alanine, protease inhibitor polypeptide sequence linked to an IκB core protein sequence according to this invention is more resistant to and has a longer half-life in the ubiquitin and proteasome-dependent degradation system than the unmodified IκB core protein.

In Vitro Protein Degradation Assay

Degradation of ^{35}S-methionine labeled protein is performed by incubation in the presence of proteasome and ubiquitin containing rabbit reticulocyte lysates and ATP regenerating system or ATP depletion system. The efficiency of protein degradation is assessed by monitoring the radioactivity released into the trichloroacetic acid (TCA)-soluble fraction and by following the disappearance of labeled protein after separation in SDS-PAGE and visualization by Phosphoimager® (Molecular Dynamics).

Degradation of Proteins in an In Vitro (Cell Free) System

Degradation of labeled protein is performed in standard 2 hr assays and assessed by SDS-PAGE and densitometry. Background and non-specific levels of degradation are determined by incubation of the substrate protein on ice and in the presence of an ATP-depletion system. The level of degradation is calculated relative to control samples incubated on ice. ATP-dependent degradation is calculated as the difference between the degradation of the substrate in the presence of ATP-regenerating and ATP-depletion systems. In parallel assays, the level of degradation is also assessed by TCA precipitation of the samples as the ratio between TCA soluble and total protein incorporated radioactivity.

PREPARATION OF RECOMBINANT FUSION PROTEIN CONTAINING PRODRUG ACTIVATING ENZYME

Effect of presence of repeat sequences on enzyme stability.

The invention also is applicable to stabilization of enzymes by insertion of a repeat sequence as defined herein into the gene encoding the enzyme and subsequent expression of the resultant fusion protein. Examples of such enzymes include prodrug-activating enzymes: for example, the herpes virus type 1 thymidine kinase gene product activates prodrugs such as FIAU and ganciclovir. Alternatively, the bacterial nitroreductase (NTR)-encoding gene encodes an enzyme, nitroreductase, which activates certain nitro compounds such as the antitumor drug CB1954 to a potent cytotoxic agent.

Recombinant Activating Polypeptides and Prodrugs Useful According to the Invention

A chimeric gene encoding the protein or polypeptide capable of activating a prodrug in the invention may encode any one of a variety of fusion proteins or polypeptides that retain the biological activity of the native protein in converting a prodrug to an active drug that is toxic to cells. Genes encoding prodrug activating proteins or polypeptides include, but are not limited to: herpes simplex viral thymidine kinase (Genbank Accession No. J02224), carboxypeptidase A (Genbank Accession No. M27717), α-galactosidase (Genbank Accession No. M13571), β-glucuronidase (Genbank Accession No. M15182), alkaline phosphatase (Genbank Accession No. J03252 J03512), or cytochrome P-450 (Genbank Accession No. D00003 N00003). Other proteins include plasmin, carboxypeptidase G2, cytosine deaminase, glucose oxidase, xanthine oxidase, β-glucosidase, azoreductase, or γ-glutamyl transferase. Preferably, the polypeptide capable of activating a prodrug is DT diaphorase or a nitroreductase. The stabilizing polypeptide may be inserted into the amino-terminal or carboxy-terminal encoding end of the gene, as described herein below for nitroreductase, or it may be inserted into a region of the gene which will not result in production of

a biologically inactive fusion protein.

Prodrugs subject to activation according to the invention are limited to those inactive prodrugs which are activatable by a polypeptide or activating enzyme encoded by and expressed from one or more genes on a vector in the target host cell. Examples of prodrugs that may be used in various embodiments of this invention include, but are not limited to, peptidyl-p-phenylenediamine-mustard, benzoic acid mustard glutamates, ganciclovir, 6-methoxypurine arabinonucleoside (araM), 5-fluorocytosine, glucose, hypoxanthine, methotrexate-alanine, N-[4-(α-D-galactopyranosyl) benyloxycarbonyl]-daunorubicin, amygdalin, azobenzene mustards, γ-glutamyl p-phenylenediamine mustard, phenolmustard-glucuronide, epirubicin-glucuronide, vinca-cephalosporin, phenylenediamine mustard-cephalosporin, nitrogen-mustard-cephalosporin, phenolmustard phosphate, doxorubicin phosphate, mitomycin phosphate, etoposide phosphate, palytoxin-4-hydroxyphenyl-acetamide, doxorubicin-phenoxyacetamide, melphalan-phenoxyacetamide, cyclophosphamide, ifosfamide, CB1954 or analogs thereof.

The gene encoding the prodrug-activating polypeptide may encode an enzyme used in the prodrug therapy described herein, and may specifically encode a nitroreductase. The nitroreductase may be any naturally occurring, mutated or artificially produced nitroreductase which is capable of activating a prodrug by reducing a nitro group present on the prodrug to, for example, the hydroxylamino intermediate. A particularly preferred nitroreductase is the *E. coli* nitroreductase (nucleotide sequence described WO 93/08288). Other nitroreductases contemplated for use according to the invention include the nitroreductases sequences described in the PCT publication number WO 93/08288. The *E. coli* B and K12 nitroreductase sequences are identical and described in EMBL Accession No. U07860. The Enterobacter nitroreductase sequence is described in Bryant *et al*, (1991), J. Biol. Chem., 266, 4126. The nucleotide sequence of the *S.*

typhimurium nitroreductase is described by Watanabe *et al*, (1990), Nucleic Acids Res., 18, 1059. A nitroreductase useful according to the invention is preferably capable of reducing one or both of the nitro groups of CB1954 and analogs thereof, to a cytotoxic form, for example, hydroxylamine.

Vector Targeting to Cells or Cell Population

Numerous techniques are known and useful according to the invention for delivering vectors and nucleic acids to target cells including the use of nucleic acid condensing agents, electroporation, complexation with asbestos, polybrene, DEAE cellulose, Dextran, liposomes, lipopolyamines, polyornithine, particle bombardment and direct microinjection (reviewed by Kucherlapati and Skoultchi, (1984), Crit. Rev. Biochem. 16, 349-379; Keown *et al*, (1990), Methods Enzymol., 185, 527).

It is contemplated according to the invention that a vector containing a gene encoding a prodrug-activating polypeptide may be delivered to a host cell non-specifically or specifically (i.e., to a designated subset of host cells) via a viral or non-viral means of delivery. Preferred delivery methods of viral origin include viral particle-producing packaging cell lines as transfection recipients for the vector of the present invention into which viral packaging signals have been engineered, such as those of adenovirus, herpes viruses and papovaviruses. Preferred gene delivery means and methods of non-viral origin that may be used in the invention include: nucleic acid condensing peptides; encapsulation in liposomes; and transfection of cells *ex vivo* with subsequent reimplantation or administration of the transfected cells. The activating enzyme can be specifically targeted to the desired site of action of the drug by linking the enzyme to a targeting ligand such as a monoclonal antibody.

Peptides derived from the amino acid sequences of viral envelope proteins have been used in gene transfer when co-administered with polylysine DNA complexes (Plank et al. (1994) J. Biol. Chem. 269, 12918-24). Trubetskoy et al. ((1992) Bioconjugate Chem. 3, 323-27 and WO 91/17773 and WO 92/19287), and Mack et al. ((1994) Am. J. Med. Sci. 307, 138-143) suggest that co-condensation of polylysine conjugates with cationic lipids can lead to improvement in gene transfer efficiency. PCT publication number WO 95/02698 discloses the use of viral components to attempt to increase the efficiency of cationic lipid gene transfer.

Nucleic acid condensing agents useful in the invention may be selected from the group consisting of spermine, spermine derivative, histones, cationic peptides and polylysine. Spermine derivative refers to analogs and derivatives of spermine and include compounds as set forth in International Publication No. WO 93/18759 (published September 30, 1993).

Disulfide bonds have been used to link the peptidic components of a delivery vehicle (Cotten et al. (1992) Meth. Enzymol. 217, 618-644); see also, Trubetskoy et al. (supra).

Delivery vehicles for delivery of DNA constructs to cells are known in the art and include DNA/polycation complexes which are specific for a cell surface receptor, as described in, for example, Wu and Wu (1988) *J. Biol. Chem* 263:14621; Wilson et al. (1992) *J. Biol. Chem.* 267:963-967; and U.S. Patent No. 5,166,320, and, for example, USSN 60/011,531, assigned to the same assignee and hereby incorporated by reference. In this co-pending application, a self-assembling virus-like particle is described and includes the DNA of interest and condensing peptides which are heteropeptides with respect to their amino acid composition (i.e., containing at least two different amino acids which are preferably basic and thus good DNA binding and DNA condensing peptides) and which have low polydispersion (i.e., a given preparation of a heteropeptide

which has low polydispersion contains peptides of very similar, if not identical lengths, such that the preparation is essentially monodispersed).

Use of Nitroreductase as a Prodrug Activating Enzyme According to the Invention

A preferred prodrug and activating enzyme combination is the 2, 4-dinitro-5-ethyleneiminobenzamide (CB1954) and nitroreductase combination. CB1954 has been shown to have an inhibiting effect on the growth of the Walker Rat Carcinoma this prodrug undergoes nitroreduction to a form a compound which is much more reactive with DNA than the prodrug, resulting in enhanced toxicity and mutagenicity (see Venitt *et al.*, (1987), Mutagenicity, 2 (5), 375-381). The use of CB1954 and analogs thereof as a prodrug in combination with a nitroreductase, is described in PCT publication number WO 93/08288.

Drabek et al. (1997) Gene Therapy (1997) 4, 101-110 have demonstrated that CB1954 treatment of transgenic mice expressing the NTR gene driven by the human CD2 locus control region results in specific cell killing in the thymus and spleen. This system is believed to be applicable according to the invention in anticancer therapy, by delivery of the NTR gene containing a repeat sequence described herein into specific target tissues, followed by administration of the CB1954 drug. Stabilization of the NTR enzyme according to the invention is believed to enhance the overall cell killing potential of the available CB1954.

We describe herein an NTR fusion protein containing the full-length (235 amino acid) Gly-Ala repeat sequence from the EBNA-1 gene. Fusions both N- and C- terminal to the NTR open reading frame are created such that the translational frame of the fusion protein is maintained. The fusion constructs are tested to establish if the repeat sequences confer enhanced stability to the NTR gene product while

retaining biological activity. In addition, reduction of repeat size and sequence variation of the repeat is analyzed by creating oligos to the consensus sequence.

Construction of NTR/repeat sequence fusions

The full-length repeat sequence of EBNA1, from the prototype B95.8 EBV strain, was cloned onto the N-terminal end of the NTR open reading frame, as follows.

The *E. coli* NTR gene was excised as a HindIII-EcoRI fragment from the pCR3-NTR-B/r plasmid (generated as a PCR fragment utilizing the Porton Down nitroreductase construct described by Drabek et al, 1997 supra, as a template, with primers as follows;

5'-GTAAGCTTGCCGCCAGCCATGGATATCATTTCT-3';

3'-TTAACCGAAGTGTAATTCGCGGCCGCAC-5') and cloned into the EcoRI/HindIII sites of pSK plasmid (Stratagene), to give pSK-NTR.

Figure 17 shows construction of a gene encoding a fusion protein according to the invention. Construction of the vector pJG-N-NTR is shown in Fig. 17. The full-length Gly-Ala repeat was excised as a BamHI-EcoRI fragment from the plasmid pBSf1GA1. The EcoRI site was chewed back using Mung bean nuclease and the BamHI site filled-in using Klenow DNA polymerase. This fragment was then cloned into the EcoRV site adjacent to the ATG codon of the NTR gene of the pSK-NTR plasmid, to give a fusion product of Gly-Ala-NTR. This was then cloned as a HindIII-NotI fragment into the eukaryotic expression vector pJG3, enabling transfection and expression in mammalian cells. pJG3 represents a CMV driven vector that contains the β-globin IVS and polyadenylation sequences and a puromycin resistance gene. The prototype pJG1 plasmid with a neomycin resistance gene is described by Drabek et al., 1997, supra). In addition, the constructs have been cloned into the pSP64Poly(A) vector (Promega). This provides an optimal vector for in vitro

gene encoding a fusion protein in which the stabilizing polypeptide is attached at the carboxy terminal end of the protein. The full-length 235 amino acid Gly-Ala repeat from pBSf1GA1 also was cloned onto the C-terminal end of the NTR gene, see Figure 18. In order to create a fusion product, the endogenous stop codon of NTR was first removed by Bal31 nuclease digestion and the resultant deletion mutant cloned into the EcoRV site of pSK. The second step was to engineer a stop codon into the pBSf1GA1 plasmid. This was done by cloning an EcoRI compatible linker into the EcoRI site of pBSf1GA1, to give pf1GA-STOP3. The linker as shown carries all three stop codons in-frame and a BamHI restriction site. The GA repeat was then excised as a BamHI cassette, blunt-ended and cloned into the EcoRI site (Klenow treated to fill-in over-hang) of pSK-NTR 2.4, to give pNTR-C. The NTR-GA fusion gene was then excised as a HindIII-NotI fragment and cloned into the eukaryotic expression vector pJG3. In addition, the constructs were cloned into the transcription/translation vector pSP64Poly(A).

The junction regions were sequenced to confirm maintenance of the reading frame. In vitro transcription/translation analyses are on-going as well as Western blotting of the expressed products.

Testing the effect of the repeats on protein stability.

The effect of protein stability is initially tested in vitro, by transient transfection of the GA-NTR constructs in COS7 cells, utilizing the plasmid pTX0147 as a control (parental NTR driven by CMV and containing the β-globin IVS and polyadenylation sequences) . At appropriate time points, the cells are immunostained with a polyclonal antibody to NTR. A comparison with parental NTR is made so as to estimate whether the GA repeat stabilizes the NTR protein in vitro. In theory, the Gly-Ala tagged constructs should offer prolonged detection by immunostaining in the

transient assay as compared to the parental NTR construct.

The transfected cells also are subjected to CB1954 treatment, to establish whether the NTR-GA fusion proteins retain their biological function in prodrug conversion and directed cell kill.

An additional measure of protein stability is made by direct injection of the plasmid constructs into tumors. For this assay, tumors are grown on the flanks of syngeneic mice by injection of 10^6-10^7 viable tumor cells (most likely the mouse squamous head and neck tumor cell line , KLN205). 10-100 μg of plasmid DNA are injected into the solid tumor mass at an appropriate stage of tumor development. After a certain period of days, the tumors are harvested and sectioned. Histological sections of the tumors are immunostained for NTR expression, and "stability" (prolonged detection of NTR in vivo) in comparison to the parental pTX0147 plasmid is appraised.

In addition, the constructs are assessed by the in vitro proteolytic degradation assays as described herein. The fusion protein will be incubated in the presence of a cell-free rabbit reticulocyte lysate containing the components of the ATP-dependent, proteasome and ubiquitin cascade system. The percent protein degraded over time can be assessed by either separation of the proteins on SDS-PAGE followed by densitometry or analysis of trichloroacetic acid (TCA) precipitation of proteins.

Testing minimization and sequence variation of the repeat motif

To assess the role of minimal motifs in stabilizing the NTR gene product, oligos based on the ZGA1 and 2 sequences were annealed together. These annealed oligos were then ligated so as to form multimers of 8 amino acids, the products of which were gel purified by PAGE and ligated into the EcoRV site of pKS.

Figure 19 shows the predicted amino acid sequence of the junction region of two of the Gly-Ala minimal repeat constructs with 25 and 33 glycine/alanine residues, respectively, in the stabilizing polypeptide. The sequences were also cloned in the reverse orientation as shown in Figure 20. Fig. 20 shows the predicted amino acid sequence of the junction region of two of the Pro-Ala minimal repeat constructs with 17 and 25 proline/alanine residues, respectively, in the stabilizing polypeptide.

The effect of these minimal motifs of both Gly-Ala and Pro-Ala, are assessed by cloning these in-frame at the N-terminal as well as the C-terminal end of the NTR gene. The resultant fusion proteins are assayed as described above.

PREPARATION OF RECOMBINANT IκB FUSION PROTEIN

Preparation of Gly-Ala coding cassettes.

The full length Gly-Ala coding region (GA) was excised from the EBNA1 containing plasmid pBSEBNA1 using AvaII and MspI restrictases. The ends were filled in by Klenow enzyme and the blunt-ended fragment was cloned into the pBS KS (+) vector previously linearized by SmaI. The orientation of the insert was estimated by dideoxysequencing from two directions, using both, universal, and reverse sequencing primers. The resulting plasmid pBSFLGA1 containing full length GA repeat is shown in Figure 4.

The derivative plasmid pBSFLGA2 (Figure 6) contains an additional ClaI site upstream of the 5' end of GA coding region and lacks the BamHI site in the same position. It was created by BamHI digest of pBSFLGA1, filling the cohesive termini by Klenow enzyme and ligating the blunt termini. The constructs allow cloning of the GA coding region in different sites of a target gene. Six possible frames could

be obtained as is shown in Figure 7.

The pBS-6xHis-FLGA-stop plasmid that contains a histidine tag at the N-end of the fusion protein. The target gene can be inserted into the BamHI site of the vector resulting in frame fusion of GA at the carboxy-terminal end. Alternatively, the EcoRI-HindIII multiple cloning site can be used. The 6xHis tag can be used for the rapid checking of frames by expression in E. coli and rapid specific purification on Ni beads. After checking, the resulting fusion-coding gene can be recloned in any eucaryotic vector. The plasmid pBS-M-FLGA which contains a Met codon immediately upstream of the GA sequence can be used for expression of N-terminal FLGA fusion proteins.

Construction of IκB-α chimeras (gene fusions).

IκB-α chimeras were constructed by inserting two types of repeat sequences at the N- or C-terminal ends of the protein. The unique alI site of the 3HA-IkB coding sequence was used for N-end insertion between the hemagglutinin tag (3HA) and the start of IκB (DiDonato et al., *Mol. Cell. Biol., 16*: 1295, 1996) (Figure 8). The glycine-alanine repeat sequence was excised from the pBS-flGA1 plasmid by BamHI and ClaI digestion and then treated with Klenow enzyme (KE) to obtain the frame (0;+2). The resulting fragment was inserted in the pBS-3H-IkB plasmid which had been cleaved with SalI followed with the filling in the 5' protruding ends by KE resulting in blunt ends with the frame (0;-1). Insertion in direct orientation resulted in a chimera (gene fusion) to express the full length Gly-Ala repeat of EBNA1 while insertion in reverse orientation resulted in a chimera for expressing a Pro-Ala repeat (Figure 9).

For C-end insertion, the 3HA-IkB was PCR amplified using primers containing an artificial XbaI site resulting in destruction of the stop codon of IκB. The PCR product was cloned in pBS KS II(+) and sequenced. The resulting plasmid

pBS-3HA-IkB(XbaI+) was opened by XbaI cleavage and treated by mung bean nuclease (MBN) to obtain the coding frame (-1;+1). A BamHI/EcoRV flGA insert was excised from the pBS-flGA1 plasmid, treated with MBN and cloned into it. The resulting plasmids pBS-3HA-IkB-C-flGA and pBS-3HA-IkB-C-flPPA encode 3HA-IκB protein with the C-end fused Gly-Ala and Pro-Ala repeats, respectively.

Testing the effect of the repeats on in vivo signal-dependent and in vitro IκB-α degradation.

To study the influence of Gly-Ala repeat on the proteasome dependent degradation of IκB, the chimeras (described above) were cloned in the pSP64polyA and pRc/CMV plasmid vectors (Figure 10). The pSP64polyA vector was used for in vitro transcription/translation and the proteins so obtained were used for in vitro degradation experiments. The pRc/CMV was used for the transfection of HeLa cell line to evaluate the signal-dependent degradation of IκB.

A representative experiment showing the in vitro degradation of the IκBα-N-flGA and control IκB-α proteins is shown in Figure 11. Experimental procedure: SP64 vectors (Promega) expressing the IκB-α chimeras were used as templates for in vitro transcription/translation procedure using the TNT coupled rabbit reticulocyte lysate system (Promega, Madison, Wisconsin). Degradation of ^{35}S-methionine labeled protein was performed by incubation in the presence of proteasome and ubiquitin containing rabbit reticulocyte lysates and ATP regenerating system or ATP depletion system. The efficiency of protein degradation was assessed by monitoring the radioactivity released into the TCA soluble fraction and by following the disappearance of labeled protein after separation in SDS-PAGE and visualization by PHOSPHOIMAGER® (Molecular Dynamics). Degradation was performed in standard 2 hrs assays. Background and non-specific levels of degradation were determined by incubation of the substrate on ice (Figure 11, control) and in the presence of ATP-

depletion system (ATP-). The level of degradation was calculated as the difference between the degradation of the substrate in the presence of ATP-regenerating and ATP-depletion systems. In parallel assays, the level of degradation was assessed by TCA precipitation of the samples as the ratio between TCA soluble and total protein incorporated radioactivity. Between 15 to 25% specific ATP-dependent degradation was detected in repeated experiments performed with in vitro translated IκB-α while no degradation was observed with the IκBα-NflGA chimera

The effect of the repeats on signal-dependent degradation of IκB-α was studied in vivo after transient transfection of the pRc/CMV based vectors in Hela cells (Figure 12). Recombinant pRc/CMV vectors expressing the 3HA-IkB, 3HA-IkB-N-lfGA and 3HA-IkB-C-FLGA chimeras were transfected in Hela cells using a LIPOFECTAMIN transfecting liposome reagent (Life Technologies, Gaithersburg, Maryland). After incubation at 37°C for 48 hours, half of the cells were treated for 20 minutes with 10 ng/ml tumor necrosis factor-α (TNF-α , Boehringer Mannhein Biochemicals, Indianapolis, Indiana) immediately before harvest in SDS-PAGE loading buffer. The cell extracts were run in 8% polyacrylamide gels, transferred to nitrocellulose filers and probed with an anti-HA mouse monoclonal antibody (Boeringher Mannheim Biochemicals, Indianapolis, Indiana). The endogenous (not shown) and 3HA-tagged IκB-α are completely degraded in transfected Hela cells following treatment for 20-30 min with 10 ng/ml TNF-α (DiDonato et al., *Mol. Cell. Biol. 16*: 1295, 1996). In contrast, the 3HA-IkB-N-lfGA and 3HA-IkB-C-FLGA chimeras (indicated in the figure by arrows) were virtually insensitive to the treatment.

Additional experiments were performed as follows.

Reagents. Human recombinant tumor necrosis factor-a (TNF-a) was obtained from Boehringer Mannheim Biochemica. The proteasome inhibitor carbobenzoxy-Leucil-Leucil-Leucinal

(MG132) and N-Acetyl-Leucil-Leucilnorieucinal (A-LLnL-H) were purchased from Peptides International. All peptide aldehydes were dissolved in DMSO and stored at -70°C. Antibodies used in this study are: anti-IkB rabbit immune serum (at dilution 1:2500 (kindly provided by J. DiDonato), anti-hemagglutinin mouse antibody 12CA5 at 2mg/ml (Boehringer Mannheim Biochemica) anti-ubiquitin rabbit immune serum at dilution 1:800 ( )

Plasmids. Wild type IkB gene tagged with three hemagglutinin epitopes 3HA-IkB was cloned in pSP64polyA vector (Promega) and used as an original construct for protein engineering. The plasmid was previously modified to eliminate the additional SalI site in the polylinker to facilitate subsequent cloning steps. The full length Gly-Ala domain of the B95.8 EBNA1 (flGA, coordinates 108201-108930) was subcloned into the SmaI site of the pBS-KS(+)II vector and was used for the insertion of flGA encoding sequence into the 3HA-IkB gene. The flGA cassette was inserted in frame into SalI site of 3HA-IkB filled by the Klenow enzyme, resulting in flGA-N chimera. For fIGA-C construct, the PCR copy of 3HA-IkB was used. The artificial XbaI site was created at pos. 907 bp. IkB gene and the natural stop-codon was destroyed using PCR mutagenesis. The resulting gene was cloned in pSP64polyA, cleaved with XbaI, the site was blunted by mung bean nuclease and the flGA existed from flGA cassette in corresponding phase was cloned downstream of the 3HA-IkB C-end encoding region. The insert fragments for chimeric genes with 24 amino acid long inserts, GG24-N, GG24-X and GG24-N have been constructed by *in vitro* synthesis using primer annealing. Primers were purchased from Gibco BRL:

5'GAGGCTCGAGGTGGTGGGGCTGGTGCTGGTGGTGCTGGAGGGGCGGGGGGG-3' and

5' TAT TGTCGACCTGCTCCTGCTCCTGCCCCTCCTGCTCCTGCCCCCCCCGCCC3',

for GA24-N;

5'GAGCTCGAGGGTGGTGGGGCTGGTGCTGGTGGTGCTGGAGGGGCGGGGGGG3'and

5'TTTGTCGACTGCTCCTGCTCCTGCCCCTCCTGCTCCTGCCCCCCCCGCCCC3';

for GA24X;

5'GAGGCTCGAGGTGGTGGTGGTGGTGGTGGTGGTGGTGGGGGGGGCGGC3'and
5'TTGTTGTCGACCTCCTCCTCCTCCTCCTCCTCCTCCTCCGCCGCCCCCCCC3'
for GG24-N.

After annealing of the 12 bp long 3' overlapping regions and filling in the 5' protruding ends by Klenow enzyme, the resulting fragments should contain SalI and XhoI sites on the opposite ends. The fragments were cleaved with SalI and Xhol and inserted in SalI or XhoI sites of 3HA-IkB gene. All chimeric constructs have been mapped with restrictases to select direct orientations and sequenced by dideoxy method (AutoRead kit, ALF sequencing machine, Pharmacia, Sweden). IkB chimeric genes as well as 3HAIkB gene have been cloned in pRc/CMV (Invitrogen) for transient expression using HindIII/XbaI sites.

Cells and transfections. HeLa cells (human cervix epithelial carcinoma line) were grown in Iscovec's modified Dulbecco's medium (GibcoBRL) supplemented with 2 mM glutamine and 10% foetal calf serum. The cells were transfected with 0.05 - 0.2 % w/v plasmid DNA and 0.6 % w/v Lipofectamine (GibcoBRL) according to the procedure recommended by the manufacturer. The efficiency of transfection was estimated by cotransfection with the pTK7.2 (Therexsys Ltd.) encoding the β-galactosidase gene under control of BPV promoter. Transfected cells were fixed with glutaraldehyde 48 hours after transfection and stained with X-gal following standard protocols. The plasmid DNAs for transfections were isolated using alkaline lysis procedure and chromatography with Qiagen Macroprep kits. Transfected cells were grown for 48 h before treatment and collecting the samples. TNFα was used at 100 ng/ml. Treatment with proteasome inhibitors N-LLnL (10 nM) or/and Z-LLL-H (10 nM) was carried out for 2 hours. Total cell extracts were prepared as follows: after treatment, the cells were immediately placed on ice and washed with ice-cold phosphate-buffered saline and SDS-PAGE sample buffer was added directly on the cell monolayer. The extracts were boiled for 10 min, sonicated and diluted in sample buffer to

a concentration of $3x10^6$ cells per ml.

Pulse-chase experiments. Transfected cells were cultured for 48 hrs in 10 cm Petri dishes and then washed twice with PBS and further incubated for 2 hrs in 9 ml of cysteine and methionine free Dulbecco's modified Eagle medium supplemented with 5% FCS. Labelling was performed for 1 h with 100 μCi of [^{35} S]methionine/cysteine mix (Amersham), after that the cells were washed with complete media and chased for the times with complete media containing 10 mM each of cold methionine and cysteine.

Immunoprecipitation. 2.5 $x10^6$ cells were washed with PBS, lysed at 4°C in lysis buffer containing 20 mM Tris-HCl pH 7.4, 150 mM NaCl, appropriate detergent (1% digitonin, or 1% triton-X-100) (Sigma), protease inhibitors (1 mg/ml of each, aprotinin, pepstatin and leupeptin), 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM DTT, 30 mM of the phosphatase inhibitor ocadaic acid, (Calbiochem). For immunoprecipitation of ubiquitinated proteins 20 mM N-etylmaleimide (NEM, Calbiochem) was added to inhibit isopeptidases. The nuclei were pelletted down at 12000 g for 15 min and supernatant was precleared with pre-immune normal serum for corresponding antibody (mouse or rabbit) for 2 h and treated with 50 mg of protein A (Sigma) for 2 h. After centrifugation, the supernatants were treated for 1 hour with 4 ml of antiIkB-specific rabbit serum or 3 ml of anti-3HA (400 μg/ml); 50 ml (50:50 w/v) protein A-Sepharose beads (Sigma) were added and after the incubation at 4° C for 1 hour the beads were washed 5 times with the lysis buffer with corresponding detergent. The immunoprecipitated protein was dissolved in SDS-PAGE loading buffer containing β-mercaptoethanol.

Immunoblot analysis. Total cell extracts (corresponding $3x10^4$ cells/well) or immunoprecipitated proteins were resolved by 10-12% SDS-PAGE, electroblotted to Protran nitrocellulose membrane (Schleicher & Shuell) and probed

with specific antibodies. After reaction with the appropriate peroxidase-conjugated secondary antibodies the complexes were visualized by enhanced chemiluminescence (ECL, Amersham Life Science).

Morphological assessment of apoptosis. HeLa cells were grown on coversleeps placed in separate wells of 24-well plates. After 48 hrs after transfection with the indicated plasmids the cells were treated with 100 ng/ml TNFα for 18 hrs. Cells were then washed in PBS, and fixed with methanol: glacial acetic acid (1:1 w/w) followed by staining with Hoechst H33258 (30mg/ml in PBS) and mounting the preparates in glycerol/DABCO. The fields were recorded using fluorescent microscope (LEITZ-DMRB, Leica, Heidelberg) equipped with a Hamamatsu 4800 cooled CCD camera (Hamamatsu, Herrsching), processing software Image-Pro Plus (Media Cybernetics), and Adobe Photoshop was used. Seven fields per slide with an average approximately 70-100 cells in each were counted for mitotic index and morphological assessment of apoptosis (highly fluorescent, fragmented chromatin or pycnotic micronuclei). All samples were run in duplicates.

Figure 21 is a schematic representation of the chimeric IkBα constructs. (A) The coding sequence of the IkBα is shown as an open box with the five ankyrin repeats indicated by hatched boxes and the encoding region for three influenza hemagglutinin epitopes (3HA) indicated by a filled box. The lysines, K21 and K22, and serines, S32 and S36, involved in signal dependent phosphorylation and ubiquitination are indicated. A cassette encoding for the 244 amino acid long Gly-Ala repeat for the B95.8 virus EBNA1 gene (f1GA) was inserted in frame at the carboxy terminal end of the IkB sequence (-C) or at the amino terminal end immediately after the 3HA tag (-N). A cassette encoding for a 24 amino acid long Gly-Ala repeat sequence was inserted at the unique SalI and XhoI site located at the amino terminal end of the IkB sequence and upstream of the anchirine repeats, respectively. A 24 amino acid long Gly polymer was inserted at the SalI site. The chimeric genes were cloned in the

pRc/CMV expression vector and transfected in HeLa. (B) The amino acid sequences of the inserts.

Figure 22 shows signal dependent degradation of IkB chimeras containing the full length Gly-Ala repeat. The time kinetics of TNFα induced degradation of IkB was monitored in HeLa cells transfected with the 3HA-IkB construct and with 3HA-IkB chimeras containing the f1GA cassette inserted at the amino- and carboxy- terminal ends of the IkB gene (IkB-f1GA-N and IkB-f1GA-C, respectively). HeLa cells were transfected with 2 μg of the indicated plasmids and cultured for 48hr before treatment with 100 ng/ml recombinant TNFα for the indicated time. Western blots of total cell extracts were probed with an IkBα-specific rabbit serum. Ectopically expressed IkB recombinant proteins are indicated by filled arrows. Open arrows indicate the endogenous IkBα which is recognized by the specific rabbit serum and serves as an internal control for degradation. (B) The intensity of the IkB specific bands was determined by scanning of the ECL developed blots. The figures represent the ratio between the intensity of the specific band before treatment and after treatment with TNFα for the indicated time. Virtually all endogenous and 3HA-tagged IkB were degraded following treatment with TNFα for 5-10 min while the f1GA containing chimeras remained unaffected even after 30 min treatment. One representative experiment out of three is shown in the figure.

Figure 23 shows signal dependent degradation of IkB chimeras containing a 24 amino acid long Gly-Ala repeat. HeLa cells transiently expressing GA24-N and GA24-X (filled arrows) were treated with TNFα and the degradation of endogenous and ectopic IkB was monitored by Western blot and densitometry as described in Figure 22.

Chimeric IkB proteins containing the Gly-Ala repeat can bind NFkB/RelA heterodimers. In Fig. 24, RelA containing complexes were immunoprecipitated from digitonin extracts of

HeLa cells transfected with 3HA-IkB or with Gly-Ala containing 3HA-IkB chimeras using anti-p65 conjugated Sepharose beads. The immunoprecipitates were fractionated by SDS-PAGE and Western blots were probed with the monoclonal antibody 12CA5 that recognizes the influenza hemagglutinin tag.

The presence of the Gly-Ala repeat does not prevent signal-dependent phosphorylation and ubiquitination of IkB. In Fig. 25, HeLa cells transfected with GA24-X and 3HA-IkB were treated with 100 ng/ml of recombinant TNFα with or without addition of 10 nM of the peptide aldehide MG123 and LLnL to inhibit proteasome activity. (A) Transfected HeLa cells were treated with TNFα for 15 min in the presence of LLnL and the ectopic IkB was then immunoprecipitated from ocadaic acid containing extracts using the anti-3HA mAbs. Western blots were probed with the anti-IkB serum. Phosphorylated IkB species with a slower migration in SDS-PAGE are indicated by arrows. (B) Ubiquitination of 3HA-IkB and GA24-X in TNFa treated HeLa cells, TNFα treatment was performed in the presence of the peptide aldehide MG123 and LLnL. IkB was immunoprecipitated from cell extracts containing 20 mM of the isopeptidase inhibitor NEM using the anti IkB rabbit serum and Western blots were probed with the anti-HA antibody. Presence of unbiquitinated IkB is visualized by the appearance of a smear of high moleculartive weight species. One representative experiment out of three. (C) The high molecular weight IkB contains ubiquitin conjugates.

Transfected HeLa cells were treated as described above and total cell extracts were immunoprecipitated with the anti-HA mAb. Western blots were probed with an anti-ubiquitin serum.

In order to determine whether the ubiquitinated Gly-Ala-containing IkB chimeras remain capable of efficient interaction with the proteasome, HeLa cells transfected with the control 3HA-IkB and with Gly-Gly-containing or Gly-Ala-

containing chimeras were treated with TNFα in the presence of proteasome inhibitors and then lysed. The lysis buffer contained 1% digitonin and 20 mM NEM in order to preserve weak molecular interactions while preventing the activity of isopeptidases. The lysates were immunoprecipitated with the a mAb specific for the conserved proteasome α subunit (MCP21) and the immunoprecipitates were then separated by SDS-PAGE. Tagged IkB molecules were detected in Western blots using the anti-HA mAb. As illustrated by the representative experiment shown in Figure 26, the ubiquitinated Gly-Ala-containing IkB polypeptides were not targeted to the proteasome. A ladder of ubiquitinated, tagged IkB polypeptides was associated with the proteasome in HeLa cells transfected with either 3HA-IkB or with the GG24-N chimera. In contrast, no such a ladder could be detected in cells expressing the GA24-N chimera even when immunoprecipitation was performed on a lysate prepared from three times as many cells. Sequential immunoprecipitation of the cell lysates with anti-p65 antibodies and probing with the anti-HA mAb confirmed that, while p65-bound IkB was efficiently depleted from lysates of cells expressing the 3HA-IkB and GG24-N chimeras, the ubiquitinated GA24-N chimera was still present in the lysates in association with p65 (not shown). Thus, it appears that Gly-Ala-containing IkB molecules are phosphorylated and ubiquitinated while associated with NF-kB but the trimolecular complex does not serve as substrate for the 26S proteasome.

The presence of the Gly-Ala repeats inhibits the physiological turnover of IkB. In Fig. 27, transfected HeLa cells were metabolically labelled in the presence of [^{35}S]Met, and then incubated in isotope free medium for the indicated time before immnunoprecipitation with anti-3HA antibodies. (A) Molecular Dynamics Phosphorimager image of SDS-PAGE. The time of chase (min) is indicated at the top. The arrows mark the bands, 3HA-IkB and GA24-X, respectively, that have been chosen for quantitative analysis. (B) Densitometry analysis. The % residual protein was

calculated as the ratio between the intensity of the specific band at the indicated time of chase and the intensity of the band at time 0.

Treatment with TNFα induces apoptosis of HeLa cells expressing Gly-Ala containing IkB chimeras. In Fig. 28, HeLa cells were cotransfected with the indicated IkB construct and with a β-gal plasmid. After 48 hrs the transfected cells were treated with 10 ng/ml TNPα for 18 hrs and apoptotic cells were then detected by Hoechst staining. The % transfected cells was estimated by staining of β-gal positive cells (A) % apoptosis in β-gal positive cells. (B) Representative Hoechst staining illustrating the induction of apoptosis in cells expressing the Gly-Ala containing IkB chimeras.

In order to investigate this phenomenon further, repeated attempts were made to produce stable HeLa transfectants expressing the Gly-Ala-repeat-containing chimeras. The results of one of such experiments are summarised in Table 4. Stable G418 resistant clones were readily obtained following transfection of the 3HA-IkB expressing plasmid. All clones expressed tagged IkB molecules of the expected size and reactivity with the available panel of IkB specific reagents. In contrast, less than 1% of the clones harvested from the cells transfected with the Gly-Ala-repeat were capable of stable growth in G418. Analysis of IκB expression in these clones using the anti-HA mAb failed to detect any ectopic IκB polypeptide or demonstrated the expression of truncated molecules that did not react with affinity purified human antibodies specific for Gly-Ala repeats or a rabbit antiserum rised against a Gly-Ala repeat synthetic peptide (not shown). The failure to obtain stable transfectants expressing Gly-Ala-repeat-containing IkB suggests that these molecules may be directly toxic to cells. It is likely that this is due to their capacity to inhibit NF-kB activity constitutively, thereby inhibiting stress responses and pathways regulating housekeeping

functions.

Insertion of a polyglycine sequence does not affect the signal dependent degradation of IkB. In Fig. 29, time kinetics of TNFA induced degradation are shown for IkB chimeras containing a 24 amino acid long Gly-Ala repeat (GA24-N) or a polyglycine sequence of similar length (GG24-N IkB). (A) Western blot analysis of total cell lysates probed with the anti hemagglutining tag antibody 12CA5. (B) Densitometry analysis. The polyG containing lkB (GG24-N) was degraded almost completely in response to the TNFα treatment in 10 min while the Gly-Ala containing chimera was virtually resistant to degradation.

In numerous experiments described herein, chimeric constructs bearing a stabilizing sequence of 24 amino acids in length (GA24 constructs) have been assayed; however, stablizing sequences of shorter chain lengths are also of use according to the invention. Using the cloning protocol by which GA24-X or GA24-N were cloned, the following putative stabilizing sequences are incorporated into a fusion protein construct (wherein G = glycine and A = alanine);

NH_2-GAGA-COOH

NH_2-GGAG-COOH

NH_2-GGAGAGAG-COOH

NH_2-GGAGGAGAGGAGAGAG-COOH

A fusion protein constuct containing a putative stablizing sequence is employed according to the methods described herein by which the GA24 constructs are tested for inhibition of degradation by proteases.

Fusion protein containing repeat sequence of proline and alanine

The stabilizing polypeptide, in an especially preferred embodiment, comprises glycine and alanine repeats. In

another preferred composition, the stabilizing polypeptide comprises proline and alanine repeats. Insertion of the f1GA cassettes in opposite orientation relative to the EBNA4 sequence resulted in an E4-PPA chimera that contains a 238 amino acid long Pro-Ala repeat. The E4-PPA chimera was degraded less efficiently than EBNA4 although the inhibitor effect of the Pro-Ala sequence appeared to be slightly weaker than that induced by the Gly-Ala repeat. Figure 30 shows that the degradation of EBNA4 was inhibited by insertion of Gly-Ala repeats of different lengths (A) and in different positions (B). A weaker inhibition was achieved by insertion of a Pro-Ala repeat (C). Specific degradation was calculated from the release of TCA-soluble radioactivity. Mean +/- SD of 4 experiments. The inhibitory effect of the repeat was calculated relative to the specific degradation of EBNA4.

DELIVERY SYSTEMS FOR INTRODUCING RECOMBINANT DNA OR PROTEIN MOLECULES OF THE INVENTION INTO EUKARYOTIC CELLS

Delivery systems are known in the art for introduction of foreign genes into eukaryotic cells. A recombinant gene fusion according to the invention may be inserted into an appropriate delivery vehicle for delivery to a target cell. The delivery vehicle may be a viral vector or a non-viral vehicle. Where the gene fusion is first inserted into an appropriate nucleic acid vector, that vector may be a plasmid, a virus or a linear DNA fragment, as desired. The vector may be naked, complexed with proteins or packaged in a delivery system such as a liposome, virosome, or a receptor mediated complex.

It is contemplated in the invention to express in specific cells a gene fusion encoding a recombinant protein joined in-frame to a sequence encoding a stabilizing polypeptide. The gene fusion may be expressed in vivo or ex vivo as a result of introduction of genetic material containing the gene fusion into cells which include but are not limited to

stem cells, macrophages, T-cells, dendritic cells, cells of hematopoietic lineage, somatic cells and tumor cells. Techniques are known in the art for delivery of recombinant DNA to these cells. Such cells can be transfected ex vivo, i.e., after removal from the body, and then re-introduced into the body, or they can be targeted in vivo.

Transfer of a recombinant gene fusion according to the invention can be accomplished through many means, including but not limited to DNA transfection using calcium phosphate coprecipitation, fusion of the target cell with liposomes containing the gene, erythrocyte ghosts or spheroplasts carrying the gene, plasmid and viral vector-mediated transfer, and DNA protein complex-mediated gene transfer such as receptor-mediated gene transfer.

1. Targeting of Cells Ex Vivo

For introduction of DNA into a cell ex vivo, a number of protocols for the transfection of various types of cells are known in the art.

Some transfection techniques involve the isolation of stem cells from total cell populations, as described in, for example, European patent applications 0 455 482 and 0 451 611. T-cell transfection is also described in Kasid et al., (1990) *Proc. Natl. Acad. Sci. USA, 87(1)*: 473. Transfection of macrophages is described in Freas et al., (1993) *Human Gene Therapy, 4*:283, and in Krall et al., (1994) *Blood, 83(9)*: L2737. Stem cell transfection is also described in Yu et al., (1995) *Proc. Natl. Acad. Sci. USA, 92(3)*: 699; Lu et al., (1994) *Human Gene Therapy, 5*: 203; Walsh et al., (1993) *Proc. Soc. Exp. Biol. Med., 204*: 289; Weinthal et al. (1991) *Bone Marrow Transplant., 8*: 403; Hamada et al., (1991) *J. Immunol. Met., 141*: 177.

Polylysine tagged with asialoglycoprotein may be used to complex and condense DNA and target the complex to

hepatocytes (Wu and Wu, (1987) *J. Biol. Chem., 262*: 4429; US Patent 5,166,320). DNA transfer is believed to occur via the asialoglycoprotein tag specifically directing the complex to only those cells expressing the asialoglycoprotein receptor. Monoclonal antibodies also may be used to target DNA to particular cell types, as described in Machy et al., (1988) *Proc. Natl. Acad. Sci. USA, 85*: 8027 - 8031; Trubetskoy et al., (1992) *Bioconjugate Chem., 3*: 323 - 327 and WO 91/17773, WO 92/19287. Lactosylated polylysine (Midoux et al , (1993) *Nucleic Acids Res., 21*: 871 - 878) and galactosylated histones (Chen et al., (1994) *Human Gene Therapy, 5*: 429 - 435) have been shown to target plasmid DNA to cells bearing lectin receptors, and insulin conjugated to polylysine (Rosenkrantz et al., (1992) *Exp. Cell Res., 199*:, 323 - 329) to cells bearing insulin receptors.

2. Targeting of Cells In Vivo

In vivo cell targeting may be accomplished according to the invention using receptor-mediated gene transfer.

Receptor-mediated gene transfer is dependent upon the presence of suitable ligands on the surfaces of cells which will allow specific targeting to the desired cell type followed by internalization of the complex and expression of the DNA. One form of receptor-mediated gene transfer is wherein a DNA vector is conjugated to antibodies which target with a high degree of specificity for cell-surface antigens (Wong and Huang, (1987) *Proc. Natl. Acad. Sci. USA, 84*: 7851; Roux et al., (1989) *Proc. Natl. Acad. Sci. USA, 86*: 9079; Trubetskoy et al., (1992) *Bioconjugate Chem., 3*: 323; and Hirsch et al., (1993) *Transplant Proceedings, 25*: 138). Nucleic acid may be attached to antibody molecules using polylysine (Wagner et al., (1990) *Proc. Natl. Acad. Sci. USA, 87*: 3410; Wagner et al., (1991) *Proc. Natl. Acad. Sci. USA, 89*: 7934) or via liposomes, as described below.

Increased expression of DNA derived from ligand-DNA complexes taken up by cells via an endosomal route has been

achieved through the inclusion of endosomal disruption agents, such as influenza virus hemagglutinin fusogenic polypeptides, either in the targeting complex or in the medium surrounding the target cell.

Targeted gene delivery is also achieved according to the invention using a DNA-protein complex. Such DNA-protein complexes include DNA complexed with a ligand that interacts with a target cell surface receptor. Cell surface receptors are thus utilized as naturally existing entry mechanisms for the specific delivery of genes to selected mammalian cells. It is known that most, if not all, mammalian cells possess cell surface binding sites or receptors that recognize, bind and internalize specific biological molecules, i.e., ligands. These molecules, once recognized and bound by the receptors, can be internalized within the target cells within membrane-limited vesicles via receptor-mediated endocytosis. Examples of such ligands include but are not limited to proteins having functional groups that are exposed sufficiently to be recognized by the cell receptors. The particular proteins used will vary with the target cell.

Typically, glycoproteins having exposed terminal carbohydrate groups are used although other ligands such as antibodies or polypolypeptide hormones, also may be employed.

Generally, a ligand is chemically conjugated by covalent, ionic or hydrogen bonding to the nucleic acid. A ligand for a cell surface receptor may be conjugated to a polycation such as polylysine with ethylidene diamino carbodiimide as described in U.S. Patent No. 5,166,320. DNA may be attached to an appropriate ligand in such a way that the combination thereof or complex remains soluble, is recognized by the receptor and is internalized by the cell. The DNA is carried along with the ligand into the cell, and is then expressed in the cell. The protein conjugate is complexed to DNA of a transfection vector by mixing equal mass

quantities of protein conjugate and DNA in 0.25 molar sodium chloride. The DNA/protein complex is taken up by cells and the gene is expressed.

Liposomes have been used for non-viral delivery of many substances, including nucleic acids, viral particles, and drugs. A number of reviews have described studies of liposome production methodology and properties, their use as carriers for therapeutic agents and their interaction with a variety of cell types. See, for example, "Liposomes as Drug Carriers," Wiley and Sons, NY (1988), and "Liposomes from Biophysics to Therapeutics," Marcel Dekker, NY (1987). Several methods have been used for liposomal delivery of DNA into cells, including poly-L-lysine conjugated lipids (Zhou et al., *Biochem. Biophys. Acta., 1065*: 8 - 14, (1991)), pH sensitive immunoliposomes (Gregoriadis, G., *Liposome Technology*, Vol I, II, III, CRC, (1993)), and cationic liposomes (Felgner et al., *Proc. Natl. Acad. Sci. USA, 84*: 7413 - 7417, (1987)). Positively charged liposomes have been used for transfer of heterologous genes into eukaryotic cells (Felgner et al., (1987), *Proc. Natl. Acad. Sci. USA, 84*: 7413; Rose et al., (1991) *BioTechniques, 10*: 520). Cationic liposomes spontaneously complex with plasmid DNA or RNA in solution and facilitate fusion of the complex with cells in culture, resulting in delivery of nucleic acid to the cell. Philip et al., (1994) *Mol. Cell. Biol., 14*: 2411, report the use of cationic liposomes to facilitate adeno-associated virus (AAV) plasmid transfection of primary T lymphocytes and cultured tumor cells.

Delivery of an agent using liposomes allows for noninvasive treatment of diseases. Targeting of an organ or tissue type may be made more efficient using immunoliposomes, i.e., liposomes which are conjugated to an antibody specific for an organ-specific or tissue-specific antigen. Thus, one approach to targeted DNA delivery is the use of loaded liposomes that have been made target-specific by incorporation of specific antibodies on the liposome

surface.

3. Viral Vector Mediated Gene Delivery

Recombinant viral vectors as well as other DNA transfer schemes can be used in practice of the present invention. A recombinant viral vector of the invention will include DNA of at least a portion of a viral genome which portion is capable of infecting the target cells and the transcription unit and control DNA sequence. By "infection" is generally meant the process by which a virus transfers genetic material to its host or target cell. Preferably, the virus used in the construction of a vector of the invention is also rendered replication-defective to remove the effects of viral replication on the target cells. In such cases, the replication-defective viral genome can be packaged by a helper virus in accordance with conventional techniques. Generally, any virus meeting the above criteria of infectiousness and capabilities of functional gene transfer can be employed in the practice of the invention.

Suitable viruses for practice of the invention include but are not limited to, for example, adenoviruses, adeno-associated virus, retroviruses, and vaccinia viruses, representative examples of which follow.

Insertion of a recombinant gene fusion of the invention into a viral vector involves conventional cloning sequences know to those of skill in the art.

Viral systems exploit the infectious capacity of viruses to introduce foreign DNA to eukaryotic cells with high efficiency. The gene of interest is cloned into the viral genome under the control of a promoter that is expressible in the selected host cell or organism. Recombinant vaccinia viruses, retroviruses and adenoviruses have often been used for DNA delivery purposes. A viral delivery vehicle is selected based on know advantages and disadvantages of its

use. One of skill in the art will select a delivery vehicle weighing such by considerations in view of the intended therapeutic use of the recombinant protein.

For example, integration of the viral vector into the host cell genome occurs after infection with a recombinant retrovirus. Such integration results in advantageous long-term expression of the transduced gene. However, retroviruses are limited in their use as vectors in that they infect only mitotic cells. Recombinant adenoviral vectors have the capacity to infect a wide spectrum of mitotic and postmitotic target cells but are maintained in the cell in episomal form, which may result in loss of the transduced gene in parallel with the cellular turnover. Recombinant vaccinia viral vectors can confer very high levels of expression of the transduced gene, and can infect a broad spectrum of target cells. However, this infection is often productive, resulting in rapid clearance of virus infected cells.

TESTING FUSION PROTEINS AND DNA MOLECULES FOR THERAPEUTIC ACTIVITY USING ANIMAL MODELS OF INFLAMMATORY BOWEL DISEASE

A variety of animal models for inflammatory bowel disease are available which may be used to test and study the therapeutic activity of fusion proteins of this invention comprising an IκB core protein linked to a stabilizing polypeptide and DNA molecules of this invention encoding such fusion proteins. Some of the animal models for inflammatory bowel disease use genetically engineered animals, for example, ulcerative colitis in mice with a disrupted IL-2 gene (Sadlack et al., *Cell, 75*: 253 - 261 (1993)); chronic enterocolitis in IL-10 deficient mice (Kühn et al., *Cell, 75*: 263 - 274 (1993)); ulcerative colitis in T cell antigen receptor α region mutant mice, T cell antigen receptor β region mutant mice, or T cell antigen receptor β x δ double mutant mice, or in mice with a mutation in the class II major histocompatibility complex Aβ gene (Mombaets, et al., *Cell, 75*: 275 - 282 (1993)); inflammatory bowel and

other organ disease in transgenic rats expressing HLA-B27 and human β_2 microglobulin (Hammer et al., *Cell, 63*: 1099 - 1112 (1990)); inflammatory bowel disease in mice expressing a dominant negative N-caderin (Hermiston and Gordon, *Science, 270*: 1203 - 1207 (1995)); and ulcerative colitis in $G\alpha_{i2}$-deficient mice (Rudolph et al., *Nature Genetics, 10*: 143 - 150 (1995)). Another model of inflammatory bowel disease uses Tgξ26 mice, which are transgenic for the human CD3ξ gene, engrafted with T cell-depleted bone marrow cells from (C57BL/6 x CBA/J)F1 donors (Holländer et al., *Immunity, 3*: 27 - 38 (1995)).

In addition to the above examples of animal models of inflammatory bowel disease using genetically engineered animals, two models of granulomatous colitis mediated by T-helper 1 (Th1) cells have been developed. One of these models involves the transfer of a subpopulation of peripheral T cells, $CD4^+$ $CD45RB^{hi}$ T cell-deficient *scid* mice (Powrie et al., *Immunity, 1*: 553 - 562 (1994)). The other animal model uses intrarectal administration of the hapten reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS) to induce colitis in BALB/c and SJL/J mice (Neurath et al., *J. Exp. Med., 182*: 1281 - 1290 (1995)). This latter TNBS-induced model of inflammatory bowel disease was recently used to test and study the therapeutic activity of anti-IL12 antibodies (Stuber et al., *J. Exp. Med., 183*: 693 - 698 (1996)) and also antisense phosphorothioate oligonucleotides to the p65 subunit of NF-κB transcription factor (Neurath et al., *Nature Medicine, 2*: 998 - 1004 (1996)).

The following description uses the TNBS-induced colitis animal model of inflammatory bowel disease, but it is understood that any animal model, such as those mentioned above, could be used in a similar manner. For TNBS-induction of colitis, pathogen free, 2 to 4 month old SJL/J mice are intrarectally injected with a 100 μl volume containing 0.5 mg of TNBS in 50% ethanol using a 3.5F catheter fitted on a 1 ml syringe (see, Neurath et al., *J.*

Exp. Med., 182: 128 1- 1290 (1995)). Clinical signs of colitis, such as severe diarrhea, weight loss, rectal prolapse, typically appear within a week. The peak of clinical symptoms occurs at about 3 weeks after administration of TNBS and then subside after 2 months.

After administration of TNBS, mice are injected locally (intrarectally) or intraperitoneally, preferably intravenously, with a fusion protein or DNA molecule encoding a fusion protein of this invention. The mice are monitored for an amelioration or abrogation of the clinical symptoms of colitis. In addition, in order to more accurately tract the course of the disease and the therapeutic efficacy of the fusion protein or DNA molecule used, histological analysis of colon, spleen, and other organs may be performed using methods known in the art (see, for example, Neurath et al., *J. Exp. Med., 182*: 1281 - 1290 (1995)). Furthermore, T cells and lamina propria macrophages may be isolated from the mice to examine the levels of expression of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α, for example, by standard ELISA or Western blot analysis coupled with densitometry (see, for example, Neurath et al., *Nature Medicine, 2*: 998 - 1004 (1996)). Antibodies to proinflammatory cytokines are commercially available (for example, PharMingen, San Diego, California).

Fusion proteins according to the invention may be tested in any of the above-described animal models by administering the fusion protein directly to the animal and observing the animal for amelioration of any of the above-described indications of inflammatory bowel disease, or by administering a recombinant DNA encoding the fusion protein and observing the animal for signs of disease amelioration, or by creating a transgenic animal using known transgenic techniques, inducing the disease as described above, and observing the animal for amelioration of indications of inflammatory bowel disease.

USE

The fusion proteins of this invention comprise a selected protein (the core protein) linked to a stabilizing polypeptide and which has a longer half-life than the unfused core protein. Such fusion proteins are thus able to provide the beneficial activity or function of the core protein, but for a longer time than the unfused core protein.

In addition, nucleic acid sequence coding for the stabilizing polypeptide of this invention, can be used for modifying genes encoding therapeutic proteins, regulatory proteins of viral vectors, or vaccine components.

Therapeutic proteins to be expressed in mammalian cells include but are not limited to, e.g., regulator proteins such as IκB, enzymes such as prodrug activating enzymes, non-inflammatory cytokines, lymphokines, cell adhesion molecules, or tumor suppressor genes.

In one embodiment, the therapeutic gene may also be a marker. For example, tumor-specific CTLs may contain a marker gene and thus be tagged with the encoded marker protein. This embodiment of the invention is useful in those cases in which a marker protein is susceptible to protease degradation in vivo or ex vivo. Once injected into a patient, the progress of the tagged CTL to the tumor site may be followed via the marker, and the marker itself is rendered longer-lived according to the invention. The marker gene and the therapeutic gene also may be one and the same.

Structural or regulatory proteins of cytokines, such as IκB, are often susceptible to protease degradation. A recombinant fusion protein comprising an IκB core protein fused to a stabilizing polypeptide as described herein, will

retain the regulatory activity of the IκB core protein and also be more stable in the lymphocyte or macrophage due to the linkage to the protease inhibitor polypeptide. Such IκB fusion proteins are useful to treat inflammatory bowel disease where the unmodified IκB (core protein) is unable to control or inhibit the formation of NF-κB transcription factor from p65 and p50 NF-κB subunits, which in turn results in high levels of proinflammatory cytokine expression and the inflammatory disease state. The recombinant DNA gene fusions encode such IκB fusion proteins are useful in gene therapy to treat inflammatory bowel disease.

Owing to their longer half-lives, proteins of this invention also may be useful as improved reagents for use in diagnosing abnormalities or diseases. For example, imaging techniques, such as MRI, can be used in vivo, wherein a fusion protein of this invention is bound to a detectable label which is capable of in vivo localization. A fusion protein comprising a core protein having a ligand-binding site is one example of an improved diagnostic regents of this invention. Many different labels and methods of labeling protein reagents are available in the art.

DOSAGE AND PHARMACEUTICAL FORMULATIONS

A patient that is a afflicted with viral or genetic disease or pathological disease may be treated in accordance with the invention via in vivo or ex vivo methods. For example in in vivo treatments, a recombinant nucleic acid or protein of the invention can be administered to the patient, preferably in a biologically compatible solution or a pharmaceutically acceptable delivery vehicle, by ingestion, injection, inhalation or any number of other methods. The dosages administered will vary from patient to patient; a "therapeutically effective dose" is determined by the level of enhancement of function of the transferred genetic material balanced against any risk or deleterious side

effects. Monitoring levels of gene introduction, gene expression and/or the presence or levels of the encoded recombinant protein will assist in selecting and adjusting the dosages administered. Generally, a composition including a recombinant protein, as described herein, is administered in a single dose in the range of 10 ng - 100 μg/kg body weight, preferably in the range of 100 ng - 10 μg/kg body weight.

The pharmaceutical compositions of the present invention may comprise the vector or delivery composition of the present invention in association with a pharmaceutically acceptable carrier or diluent, preferably one for parenteral administration. The pharmaceutical compositions of the present invention may additionally comprise a co-factor for the polypeptide capable of activating the prodrug. The co-factor is preferably a ribonucleoside or ribonucleotide of nicotinic acid or nicotinamide, especially when the polypeptide encoded by the vector of the present invention is a nitroreductase.

Preferably, when using the vector or delivery vehicle or pharmaceutical composition of the present invention in prodrug therapy, the prodrug and the vector or delivery composition or pharmaceutical composition are administered to the patient requiring treatment as a combined preparation for simultaneous, separate or sequential use.

The prodrug and the vector or delivery composition can be administered simultaneously but it is often found preferable, in clinical practice, to administer the vector or delivery vehicle prior to administering the prodrug, e.g., up to 72 hours before, in order to give the vector an opportunity to establish itself in the target cell and to express the polypeptide capable of activating the prodrug. By operating in this way, when the prodrug is administered, conversion of the prodrug to the cytotoxic agent will be confined to the regions where the polypeptide is expressed,

i.e., the region of the target cell, and damage to healthy cells caused by the cytotoxic agent is minimized. Clinical determination of effectiveness of the inventive methods involving prodrug administration and depletion of cells of a target cell population will include indications such as reduction in tumor size, with at least a 25% reduction being considered as clinically effective treatment, or by depletion of a portion of targeted cells of a cell population, e.g., depletion of at least 10% - 50% of target cells.

The exact dosage regime will, of course, need to be determined by individual clinicians for individual patients and this, in turn, will be controlled by the exact nature of the prodrug. Prodrug chemotherapy normally involves parenteral administration of both the prodrug and the vector or delivery vehicle, and administration by the intravenous route is frequently found to be most practical.

The dosage will depend upon the disease indication and the route of administration but should be between 1-1000μg of the nucleic acid or nucleic acid component/kg of body weight/day, preferably between about 50-200 μg/kg. The duration of treatment will extend through the course of the disease symptoms, possibly continuously. The number of doses will depend upon disease delivery vehicle and efficacy data from clinical trials.

A vector or composition of the invention containing a gene encoding a prodrug-activating polypeptide may be administered via a route which includes intramuscular, intravenous, aerosol, oral (tablet or pill form), topical, systemic, ocular, as a suppository, intraperitoneal and/or intrathecal, as well as direct injection of the vector or vector/delivery vehicle complex into a tumor mass.

A vector or composition containing a gene encoding a prodrug-activating polypeptide, which is slowly dissipated

by systematic adsorption are preferred. Intravenous administration with a drug carrier designed to increase the circulation half-life of the vector or composition can be used. The size and composition of the drug carrier restricts rapid clearance from the blood stream. The carrier, made to accumulate at the desired site of transfer, can protect the nucleic acid sequence encoding an episomal vector containing a gene encoding a prodrug-activating polypeptide from degradative processes.

Controlled release drug delivery systems, such as nanoparticles and hydrogels, may be potential delivery systems for a vector containing a gene encoding a prodrug-activating polypeptide. These carriers have been developed for chemotherapeutic agents and protein-based pharmaceuticals, and consequently, can be adapted for nucleic acid delivery.

A vector or composition described herein also can be co-formulated with permeability enhancers, such as Azone, or oleic acid, in a liposome. The liposomes can either represent a slow release presentation vehicle in which the vector containing a gene encoding a prodrug-activating polypeptide and permeability enhancer transfer from the liposome into the targeted cell, or the liposome phospholipids can participate directly with the modified nucleic acid sequence encoding an episomal vector containing a gene encoding a prodrug-activating polypeptide and permeability enhancer can participate directly thereby facilitating cellular delivery. In some cases, both the vector containing a gene encoding a prodrug-activating polypeptide and permeability enhancer can be formulated into a suppository formulation for slow release.

Animal Models for Screening *In Vivo* for New Prodrugs

In another embodiment of this invention, there is provided a mammalian model for determining efficacy of treatment

methods of the invention, and for determining the efficacy of fusion proteins having increased half-lives according to the invention. The mammalian model comprises a transgenic animal whose cells contain the vector of the present invention encoding a polypeptide which is being tested in combination with a prodrug. Methods of making transgenic mice (Gordon et al., 1980, PNAS 77:7380; Harbers et al., 1981, Nature 293:540; Wagner et al., 1981, PNAS 78:5016; and Wagner et al., 1981, PNAS 78:6376) , sheep, pigs, chickens (see Hammer et al., 1985, Nature 315:680), etc., are well-known in the art and are contemplated for use according to the invention.

The transgenic animal may have a tumor or the propensity to develop a tumor. Stewart et al., (1993, Int. J. Cancer 53:1023) describe a transgenic mouse having a T-cell tumor. Teitz et al., (1993, PNAS 90:2910) describe transgenic mice having rhabdomyo sarcomas and insulin-producing pancreatic-islet tumors. A vector containing a gene encoding a prodrug activating polypeptide may be transduced into ova from any of these tumor-bearing mice to create an animal model for determining the efficacy of treatment methods of the invention involving prodrugs according to the invention. Such an animal model is also useful for *in vivo* testing of the half-life and efficacy of fusion proteins containing activating enzyme according to the invention.

A fusion protein having an increased half-life, as defined herein, will be considered more effective than the unfused native protein if treatment using the fusion protein produces more beneficial clinical effects or results in the need for administration of a smaller amount of prodrug, or produces a better reduction in tumor load.

EFFICACY IN HUMANS

The efficacy of the fusion proteins of this invention in the therapeutic treatment of inflammatory bowel disease humans

can be determined by a variety of methods. As noted above with respect to testing therapeutic activity in animal models, amelioration of many of the signs of inflammatory bowel disease can be monitored by observing a patient who has received a fusion protein or DNA encoding a fusion protein of this invention. For example, the trained health care provider can use standard methods, including direct interview and/or observation, of a patient for amelioration or elimination of diarrhea, reversal of weight loss or increase in weight, healing of intestinal lesions, and reversal of prolapsed rectal tissue where evident.

In addition, the therapeutic efficacy of a fusion protein or DNA molecule encoding such fusion protein of this invention which has been administered to a patient with inflammatory bowel disease can also be assessed using any of a number of assays that are similarly used in animal model studies. For example, endothelial cells, T cells, and/or macrophages isolated from a patient treated with a fusion protein or DNA molecule of this invention can be assayed to detect a change (preferably a decrease) in levels of one or more the NF-κB subunits p65 and p50, or one or more proinflammatory cytokines, such as IL-1, IL-6, and/or TNF-α (see, for example, Neurath et al., *Nature Medicine, 2*: 998 - 1004 (1996)) and as described below.

1. Isolation of lamina propria macrophages from patients.

Lamina propria mononuclear cells are isolated from patients using the standard method of Bull et al., *(J. Clin. Invest., 59*: 966 - 974 (1977)) and enriched for macrophages by negative selection using antibodies to CD8 (for example, the monoclonal antibody OKT8), CD4 (for example, the monoclonal antibody OKT4), and anti-erythroglycoprotein. Antibody-coated cell populations are removed by incubation with immunomagnetic beads coated with anti-murine IgG antibody (Advanced Magnetics, Cambridge, Massachusetts) followed by incubation with immunomagnetic beads coated with

anti-murine IgG antibody (Dynal, Oslo, Norway).

The macrophage-enriched lamina propria mononuclear cells from the patients are suspended and grown in an appropriate culture medium, such as complete RPMI medium consisting of RPMI 1640 supplemented with L-glutamine (3 mM), HEPES buffer (10 mM), gentamicin (10 μg/ml), penicillin and streptomycin (each at 100 U/ml), 2ME (0.005 mM), heat-inactivated fetal calf serum (10%), and with or without 40 μg/ml lipopolysaccharide (LPS) and 10 μg/ml phytohemagglutinin (PHA) (medium and supplemented components obtainable from Sigma Chemical Co., St. Louis, Missouri) to induce proinflammatory cytokine production, if desired.

2. Production of cytokines in cultured macrophages.

Levels of proinflammatory cytokines culture supernatants from macrophages are determined using antibodies to human cytokines IL-1, IL-6, and TNF-α (available, for example, from Life Technologies, Gaithersburg, Maryland and R & D Systems, Minneapolis, Minnesota) in a standard ELISA system (see, for example, Neurath et al., *Nature Medicine, 2*: 998 - 1004 (1996)).

3. Northern hybridization and mobility shift assays.

Levels of p65 and p50 subunits of NF-κB transcription factor in lamina propria macrophages can also be examined by Northern hybridizations and mobility shift assays (also called gel retardation assays).

Total macrophage RNA is isolated using a standard extraction method, such as a guanidium thiocyanate/phenol and chloroform extraction procedure. Sample of RNA (10 μg) are separated on agarose gels, then blotted onto a nitrocellulose filter (0.2 μm) and hybridized to a specific DNA probe for each selected proinflammatory cytokine (for example, IL-1, IL-6, and TNF-α), which can be generated by

RT-PCR amplification from B cell cDNA from spleen cells

For mobility shift assays, nuclear proteins are isolated from the macrophages and mobility shift assays performed to detect p65 and/or p50 binding to NF-κB DNA binding site, such as described by Neurath et al., *Proc. Natl. Acad. Sci. USA, 92*: 5336 - 5340 (1995)).

4. Western blot analysis and densitometric analysis of nuclear proteins.

Nuclear proteins are extracted from macrophages, for example, by the method of Neurath et al. (*Proc. Natl. Acad. Sci. USA, 92*: 5336 - 5340 (1995)) and blotted on nitrocellulose filters (0.45 μm) and p65 and p50 NF-κB subunits detected using rabbit anti-p65 and anti-p50 antibodies (Santa Cruz Biotechnology, Santa Cruz, California) and a detecting system such as antibody such as alkaline phosphatase-labeled goat anti-rabbit antibody and alkaline phosphatase color substrate (Promega, Madison, Wisconsin). Western blots are analyzed with a densitometer and an appropriate instrument program (for example, IMAGEQUANT, Molecular Dynamics).

While the invention has been described with respect to certain specific embodiments, it will be appreciated that many modifications and changes may be made by those skilled in the art without departing from the spirit of the invention. It is intended, therefore, by the appended claim to cover all such modifications and changes as fall within the true spirit and scope of the invention.

OTHER EMBODIMENTS

Other embodiments will be evident to those of skill in the art. It should be understood that the foregoing detailed description is provided for clarity only and is merely exemplary. The spirit and scope of the present invention are

not limited to the above examples, but are encompassed by the following claims.

Table 1 Primers for multimerization of minimal motifs

	primer sequence	minimal motif								
ZGA1	5' GGAGCTGGTGCTGGAGGTGCGGGT3'	G	A	G	A	G	G	A	G	
ZGA2	5' CTCCACCCGCACCTCCAGCACCAG3'	GCA	GCT	GGT	GCT	GCA	GGT	GCG	GGT	
			GA	CCA	CGA	CCT	CCA	CGC	CCA	CCT C
ZGS1	5' GGAAGTGGAGCTGGAGGTGCGGGT3'	G	S	G	A	G	G	A	G	
ZGS2	5' TTCCACCCGCACCTCCAGCTCCAC3'	GGA	AGT	GGA	GCT	GGA	GGT	GCG	GGT	
			CA	CCT	CGA	CCT	CCA	CGC	CCA	CCT T
ZGR1	5' GGAAGAGGAGCTGGAGGTGCGGGT3'	G	R	G	A	G	G	A	G	
ZGR2	5' TTCCACCCGCACCTCCAGCTCCTC3'	GGA	AGA	GGA	GCT	GGA	GGT	GCG	GGT	
			CT	CCT	CGA	CCT	CCA	CGC	CCA	CCT T
ZGE1	5' GGAGAAGGAGCTGGAGGTGCGGGT3'	G	E	G	A	G	G	A	G	
ZGE2	5' CTCCACCCGCACCTCCAGCTCCTT3'	GGA	GAA	GGA	GCT	GGA	GGT	GCG	GGT	
			TT	CCT	CGA	CCT	CCA	CGC	CCA	CCT C
ZGY1	5' GGATATGGAGCTGGAGGTGCGGGT3'	G	Y	G	A	G	G	A	G	
ZGY2	5' ATCCACCCGCACCTCCAGCTCCAT3'	GGA	TAT	GGA	GCT	GGA	GGT	GCG	GGT	
			TA	CCT	CGA	CCT	CCA	CGC	CCA	CCT A
ZMGS1	5' GGATCAGGCAGCGGAGGTAGCGGT3'	G	S	G	S	G	G	S	G	
ZMGS2	5' ATCCACCGCTACCTCCGCTGCCTG3'	GGA	TCA	GGC	AGC	GGA	GGT	AGC	GGT	
			GT	CCG	TCG	CCT	CCA	TCG	CCA	CCT A
ZMGR1	5' GGACGTGGCCGAGGAGGTAGAGGT3'	G	R	G	R	G	G	R	G	
ZMGR2	5' GTCCACCTCTACCTCCTCGGCCAC3'	GGA	CGT	GGC	CGA	GGA	GGT	AGA	GGT	
			CA	CCG	GCT	CCT	CCA	TCT	CCA	CCT G
ZMGE1	5' GGAGAGGGCGAAGGAGGTGAAGGT3'	G	E	G	E	G	G	E	G	
ZMGE2	5' CTCCACCTTCACCTCCTTCGCCCT3'	GGA	GAG	GGC	GAA	GGA	GGA	GAA	GGT	
			TC	CCG	CTT	CCT	CCA	CTT	CCA	CCT C

Table 2 Coding capacity of gly-ala polymers

primer set	positive orientation								negative orientation							
ZGA	gly	ala	gly	ala	gly	gly	ala	gly	leu	his	pro	his	leu	gln	his	gln
ZGS	gly	ser	gly	ala	gly	gly	ala	gly	phe	his	pro	his	leu	gln	leu	his
ZGR	gly	arg	gly	ala	gly	gly	ala	gly	phe	his	pro	his	leu	gln	leu	leu
ZGE	gly	glu	gly	ala	gly	gly	ala	gly	leu	his	pro	his	leu	gln	leu	leu
ZGY	gly	tyr	gly	ala	gly	gly	ala	gly	ile	his	pro	his	leu	gln	leu	his
ZMGS	gly	ser	gly	ser	gly	gly	ser	gly	ile	his	arg	tyr	leu	arg	cys	leu
ZMGR	gly	arg	gly	arg	gly	gly	arg	gly	val	his	leu	tyr	leu	leu	gly	his
ZMGE	gly	glu	gly	glu	gly	gly	glu	gly	leu	his	leu	his	leu	leu	arg	pro

Table 3 Cloning of gly-ala repeats into the Sma I site of pGEX-2T

i digestion of pGEX-2t with Sma I:

BamHI Sma I EcoRI
GTTGGATCCCCGGGAATTCATCGT
CAACCTAGGGGCCCTTAAGTAGCA

GTTGGATCCCC GGGAATTCATCGT
CAACCTAGGGG CCCTTAAGTAGCA

ii. modification of the ends of the gly-ala polymer with Klenow:

GGA GCT GGT N....N GCG GGT
GA CCA N....N CGC CCA CCT C

GGA GCT GGT N....N GCG GGT GGA G
CCT CGA CCA N....N CGC CCA CCT C

iii. insertion of gly-ala into the pGEX-2T Sma I site:

pGET-2T	gly-ala repeat	pGEX-2T
GTTGGATCCCC	GGA GCT GGT N....N GCG GGT GGA G	GGGAATTCATCGT
CAACCTAGGGG	CCT CGA CCA N....N CGC CCA CCT C	CCCTTAAGTAGCA

Table 4: **Recovery of stable geneticin resistant clones of HeLa cells transfected with GAr containing IκB chimeras.**

Construct	Nr. Of clones	G416 resistant clones	%	Size of expressed protein (kD)	
				Expected*	Observed
3HA-IkBα	24	18	75.0	42	42
flGA-C	48	3	0.6	75	42;46;50
flGA-N	48	2	0.4	75	30;55
GA24-X	48	3	0.6	49	40;42;43

CLAIMS:

1. A method for increasing the resistance of a core protein to proteolytic degradation, comprising linking or inserting a stabilizing polypeptide onto or into the core protein, wherein the stabilizing polypeptide has the general formula:

$$[(Gly_a)X(Gly_b)Y(Gly_c)Z]n$$

wherein each Gly_a, Gly_b, Gly_c, independently, may be one, two, three, four, five or six sequential glycine residues; each of X, Y and Z is, independently, selected from the group consisting of alanine, serine, valine, isoleucine, leucine, methionine, phenylalanine, proline, and threonine; wherein X, Y and Z, respectively, need not be identical from n repeat to n repeat;
wherein n is 1 to 66.

2. A method for increasing the resistance of a core protein to proteolytic degradation, comprising linking or inserting a nucleotide sequence encoding a stabilizing polypeptide to a nucleotide sequence encoding a core protein to create a gene fusion which is expressible as a fusion protein, wherein the stabilizing polypeptide has the general formula:

$$[(Gly_a)X(Gly_b)Y(Gly_c)Z]n$$

wherein each Gly_a, Gly_b, Gly_c, independently, may be one, two, three, four, five or six sequential glycine residues; each of X, Y and Z is, independently, selected from the group consisting of alanine, serine, valine, isoleucine, leucine, methionine, phenylalanine, proline, and threonine; wherein X, Y and Z, respectively, need not be identical from n repeat to n repeat;
wherein n is 1 to 66.

3. The method according to claim 1 or claim 2, wherein each of X, Y and Z is, independently, selected from the group consisting of alanine, serine, methionine and proline.

4. The method according to claim 3 wherein each of X, Y and Z is, independently, alanine or serine.

5. The method according to claim 3 wherein each of X and Y is, independently, serine or methionine.

6. The method according to claim 3 wherein each of X and Y is, independently, methionine or proline.

7. The method according to claim 1 or claim 2 wherein the stabilizing polypeptide comprises a 17 amino acid sequence containing glycine and alanine residues.

8. The method according to any one of the preceding claims, wherein the core protein is IκB regulator protein.

9. The method according to any one of claims 1 to 7, wherein the core protein is an enzyme.

10. The method according to claim 9 wherein the enzyme is a nitroreductase.

11. The method according to any one of the preceding claims wherein the stabilizing polypeptide has an amino acid sequence comprising the 235 amino acid sequence of Figure 1.

12. A protein produced by the method of any one of the previous claims having increased resistance to proteolytic degradation, wherein the core protein is a non-immunogenic protein.

13. A recombinant DNA molecule encoding a fusion protein according to claim 12.

14. A method of treating a disease comprising administering an effective amount of the fusion protein of claim 12.

15. The protein of claim 12 or the recombinant DNA molecule of claim 13 for use in therapy.

16. Use of the fusion protein of claim 12 in the manufacture

of a medicament to treat a disease.

17. The use of claim 16 wherein the disease is inflammatory bowel disease.

18. A method of inhibiting the dissociation of the NFκB p65 and p50 protein subunits which are held together by an IκB regulator protein comprising providing an effective amount of the protein produced by the method of claims 8.

19. A method of regulating expression of a proinflammatory cytokine whose expression requires dissociation of the NFκB p65 and p50 protein subunits which are held together by an IκB regulator protein comprising providing an effective amount of the protein produced by the method of claim 8.

20. A method of regulating a transcriptional activation pathway which requires dissociation of protein subunits held together by a regulator protein, wherein normal degradation of the regulator protein results in dissociation of the protein subunits, comprising providing a fusion protein which is capable of holding the protein subunits together and which has a greater resistance to degradation than the regulator protein, the fusion protein which comprises the regulator protein being covalently linked to a stabilizing polypeptide and which by virtue of the increased resistance to normal degradation prevents dissociation of the protein subunits for a longer period of time than the regulator protein.

21. A method of detecting an abnormality or disease state comprising labeling the protein of claim 12, administering the labeled protein to a patient, and detecting the labeled fusion protein in the patient.

22. A kit for administering treatment of a disease comprising an effective amount of the protein of claim 12 in a packaging means.

23. A kit for administering treatment of a disease comprising an effective amount of the DNA molecule of claim 13 in a packaging means.

24. A kit for administering treatment of a disease, the kit comprising, separately packaged, a vector encoding a fusion protein according to claim 12 wherein the fusion protein comprises a prodrug activating enzyme, and a prodrug.

25. A method of depleting a cell population *in vivo*, comprising
administering a prodrug to a cell population targeted for depletion and containing a gene encoding a fusion protein comprising a prodrug activating enzyme and a stabilizing polypeptide, wherein said prodrug is activated by said fusion protein and said prodrug is administered in an amount sufficient to deplete cells of said cell population upon prodrug activation.

26. The method of claim 25 wherein said prodrug activating enzyme comprises nitroreductase and said prodrug comprises CB1954.

27. A method of treating a tumor in a mammal comprising administering to the mammal a vector encoding a protein according to claim 12, wherein the protein is a prodrug activating enzyme, and a prodrug capable of activation by the prodrug activating enzyme.

28. The method of claim 27 wherein said prodrug is administered concomitant with said vector.

29. Use of a stabilizing polypeptide for increasing the resistance of a core protein to proteolytic degradation, comprising linking or inserting the stabilizing polypeptide onto or into the core protein, wherein the stabilizing polypeptide has the general formula:

$[(Gly_a)X(Gly_b)Y(Gly_c)Z]n$

wherein each Gly_a, Gly_b, Gly_c, independently, may be one, two, three, four, five or six sequential glycine residues; each of X, Y and Z is, independently, selected from the group consisting of alanine, serine, valine, isoleucine, leucine, methionine, phenylalanine, proline, and threonine; wherein X, Y and Z, respectively, need not be identical within each repeat;

wherein n is 1 to 66.

30. Use of a stabilizing polypeptide for increasing the resistance of a core protein to proteolytic degradation, comprising linking or inserting a nucleotide sequence encoding the stabilizing polypeptide to a nucleotide sequence encoding the core protein to create a gene fusion which is expressible as a fusion protein, wherein the stabilizing polypeptide has the general formula:

$[(Gly_a)X(Gly_b)Y(Gly_c)Z]n$

wherein each Gly_a, Gly_b, Gly_c, independently, may be one, two, three, four, five or six sequential glycine residues; each of X, Y and Z is, independently, selected from the group consisting of alanine, serine, valine, isoleucine, leucine, methionine, phenylalanine, proline, and threonine; wherein X, Y and Z, respectively, need not be identical within each repeat;

wherein n is 1 to 66.

FIG.1

NH_2- Gly-Ala-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala-
Gly-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-
Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-
Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-
Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-
Gly-Ala-Gly-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-
Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-
Ala-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-
Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-
Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-
Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-
Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-
Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-
Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-
Gly-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala -COOH

FIG. 2

GGATCCAAGT TGCATTGGCT GCAAAGGGAC CCACGGTGGA ACA GGA GCA GGA GCA
Gly Ala Gly Ala

GGA GCA GGA GGG GCA GGA GCA GGA GGA GCA GGA GGC AGTGGAGGCA
Gly Ala Gly Gly Ala Gly Gly Gly Ala Gly Gly

GTGGAGGCCG GGGTGCAGAA TTCGAT

gatcccccgacccacggtggaacaggagcaggagcaggagcgggaggggca

D P P T H G G T G A G A G A G G A

ggagcaggaggggcaggagcaggaggaggggcaggagcaggaggaggggca

G A G G A G A G G G A G A G G G A

ggaggggcaggaggggcaggaggggcaggagcaggaggaggggcaggagca

G G A G G A G G A G A G G G A G A

ggaggaggggcaggaggggcaggaggggcaggagcaggaggaggggcagga

G G G A G G A G G A G A G G G A G

gcaggaggaggggcaggaggggcaggagcaggaggaggggcaggaggggca

A G G G A G G A G A G G G A G G A

ggaggggcaggagcaggaggaggggcaggagcaggaggaggggcaggaggg

G G A G A G G G A G A G G G A G G

gcaggagcaggaggaggggcaggaggggcaggaggggcaggagcaggagga

A G A G G G A G G A G G A G A G G

ggggcaggagcaggaggggcaggaggggcaggagcaggaggggcaggaggg

G A G A G G A G G A G A G G A G G

gcaggagcaggaggaggggcaggaggggcaggagcaggaggaggggcaggagca

A G A G G G A G G A G A G G G A G

ggaggggcaggagcaggaggaggggcaggagcaggaggaggggcaggaggg

G A G A G G A G G A G A G G A G G

gcaggagcaggaggaggggcaggaggggcaggagcaggaggaggggcaggagca

A G A G G A G G A G A G G G A G A

ggaggggcaggagcaggaggtggaggccggggtcgaggaggcagtggaggc

G G A G A G G G G R G R G G S G G

cggggctgcaggaattcgatatcgagcttatcg

R G C R N S I S S L S

FIG. 3

FIG. 4A

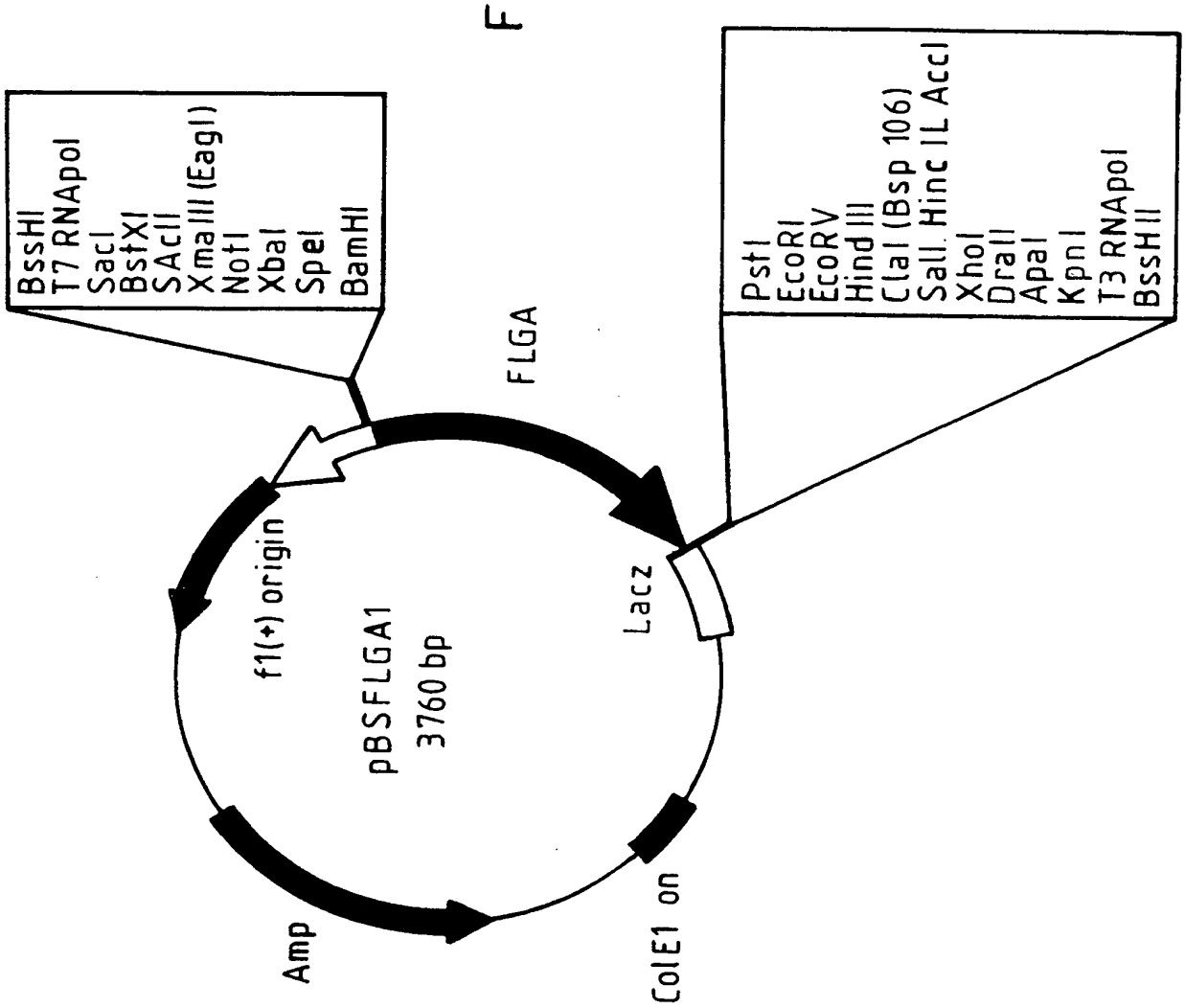

BssHI
T7 RNApol
SacI
BstXI
SAcII
XmaIII(EagI)
NotI
XbaI
SpeI
BamHI
FLGA
PstI
EcoRI
EcoRV
HindIII
ClaI(Bsp 106)
SalI. HincIL AccI
XhoI
DraII
ApaI
KpnI
T3 RNApol
BssHII
f1(+) origin
pBSFLGA1
3760 bp
Lacz
Amp
ColE1 on

FIG. 4B

pBS KS (+) T7 promoter -> SacI, NotI, XbaI

BamHI

ggatcccccgacccacggtggaacaggagcaggagcaggagcgggaggggcaggagcaggaggggcaggagcaggaggaggggcagg
agcaggaggaggggcaggaggggcaggaggggcaggaggggcaggagcaggaggaggggcaggagcaggaggaggggcaggaggg
gcaggaggggcaggagcaggaggaggggcaggagcaggaggaggggcaggaggggcaggagcaggaggaggggcaggaggggcag
gaggggcaggagcaggaggaggggcaggagcaggaggaggggcaggaggggcaggagcaggaggaggggcaggaggggcaggagg
ggcaggagcaggaggaggggcaggagcaggaggggcaggaggggcaggaggggcaggagcaggaggggcaggagcaggaggaggg
gcaggaggggcaggaggggcaggagcaggaggggcaggagcaggaggggcaggagcaggaggggcaggagcaggaggggcaggag
gggcaggagcaggaggggcaggaggggcaggagcaggaggggcaggaggggcaggagcaggaggaggggcaggaggggcaggagc
aggaggaggggcaggaggggcaggagcaggaggggcaggaggggcaggagcaggaggggcaggaggggcaggagcaggaggggca
ggaggggcaggagcaggaggaggggcaggagcaggaggggcaggagcaggag

EcoRI EcoRV ClaI

gtggaggccggggtcgaggaggcagtggaggccggggctgcaggaattcgatatcgagcttatcg

——————————pBS KS(+) vector sequence ——>

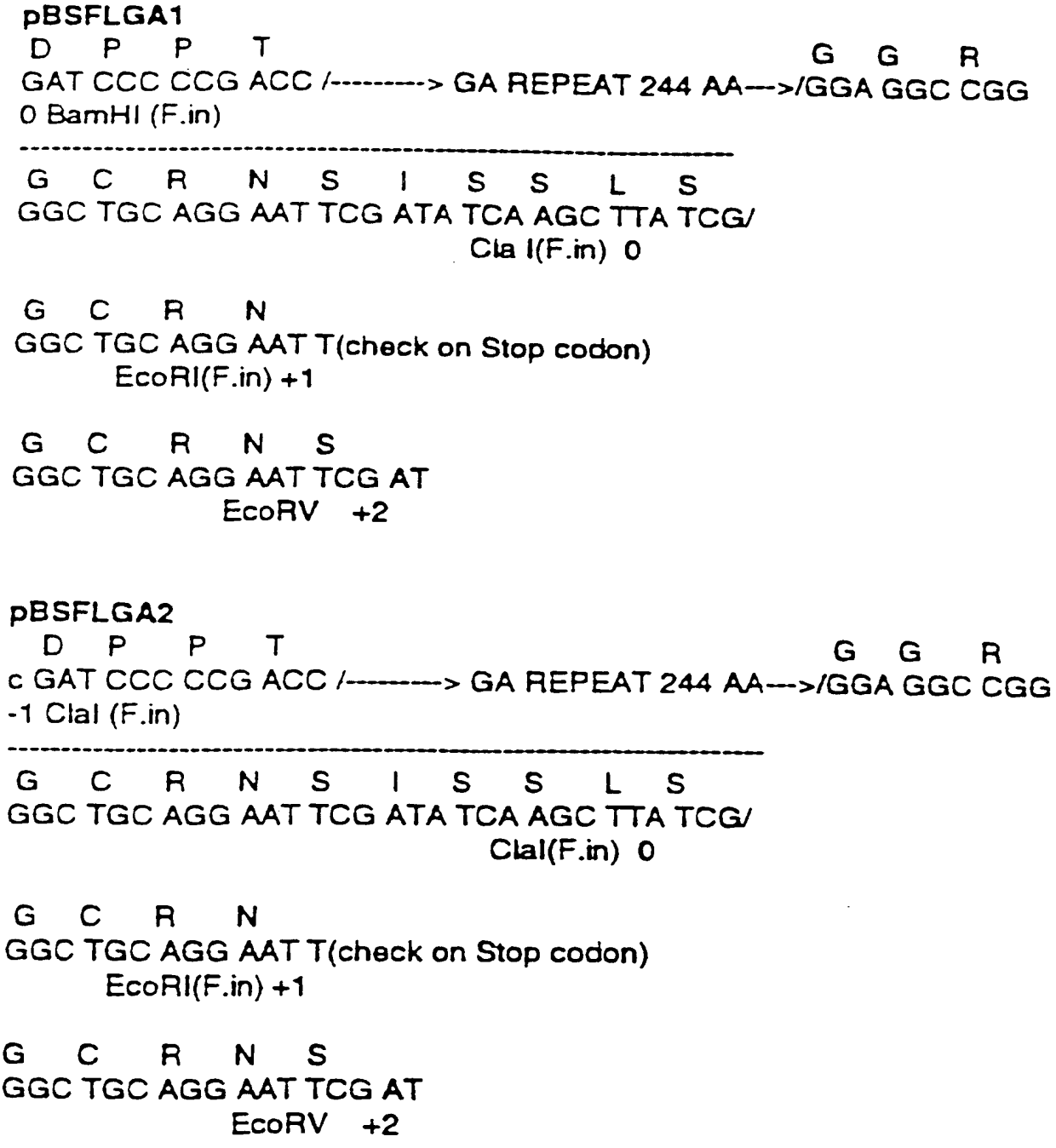
FIG. 5

pBS KS (+) T7 promoter -> SacI, NotI, XbaI
——————————————————>

ClaI
ggatcgatcccccgacccacggtggaacaggagcaggagcaggagcgggaggggcaggagcaggaggggcaggagcaggaggagggg
caggagcaggaggaggggcaggaggggcaggaggggcaggaggggcaggagcaggaggaggggcaggagcaggaggaggggcagg
aggggcaggaggggcaggagcaggaggaggggcaggagcaggaggaggggcaggaggggcaggagcaggaggaggggcaggaggg
gcaggaggggcaggagcaggaggaggggcaggagcaggaggaggggcaggaggggcaggagcaggaggaggggcaggaggggcag
gaggggcaggagcaggaggaggggcaggagcaggaggggcaggaggggcaggaggggcaggagcaggaggggcaggagcaggagg
aggggcaggaggggcaggaggggcaggagcaggaggggcaggagcaggaggggcaggagcaggaggggcaggagcaggaggggca
ggaggggcaggagcaggaggggcaggaggggcaggagcaggaggggcaggaggggcaggagcaggaggaggggcaggaggggcag
gagcaggaggaggggcaggaggggcaggagcaggaggggcaggaggggcaggagcaggaggggcaggaggggcaggagcaggagg
ggcaggaggggcaggagcaggaggaggggcaggagcaggaggggcaggagcaggag
EcoRI EcoRV ClaI
gtggaggccggggtcgaggaggcagtggaggccggggctgcaggaattcgatatcgagcttatcg

——————pBS KS(+) vector sequence ——>

FIG. 6


pBSFLGA1
D P P T G G R
GAT CCC CCG ACC /---------> GA REPEAT 244 AA--->/GGA GGC CGG
0 BamHI (F.in)
G C R N S I S S L S
GGC TGC AGG AAT TCG ATA TCA AGC TTA TCG/
Cla I(F.in) 0
G C R N
GGC TGC AGG AAT T(check on Stop codon)
EcoRI(F.in) +1
G C R N S
GGC TGC AGG AAT TCG AT
EcoRV +2
pBSFLGA2
D P P T G G R
c GAT CCC CCG ACC /---------> GA REPEAT 244 AA--->/GGA GGC CGG
-1 ClaI (F.in)
G C R N S I S S L S
GGC TGC AGG AAT TCG ATA TCA AGC TTA TCG/
ClaI(F.in) 0
G C R N
GGC TGC AGG AAT T(check on Stop codon)
EcoRI(F.in) +1
G C R N S
GGC TGC AGG AAT TCG AT
EcoRV +2

p6hisMetFLGAstop

pQE32 RBS **M** 6xHis G
========= atg aga gga tcg *** *** *** *** *** *** ggg atc c
BamHI

R M G R D P P T
gc atg ggt cga **gat ccc ccg acc**
SphI/NcoI

|————————FLGA 244 aa————————>

G C R N_
ggc tgc agg aat tcg att cga caa gga ggt acc ccg ggt cga cct
EcoRI KpnI SmaI SalI PstI
gca gcc aag ctt aat tag ctg ag
HindIII 3 x stop

pMetFLGA

pBS KS(+) ggg atc c
========== BamHI

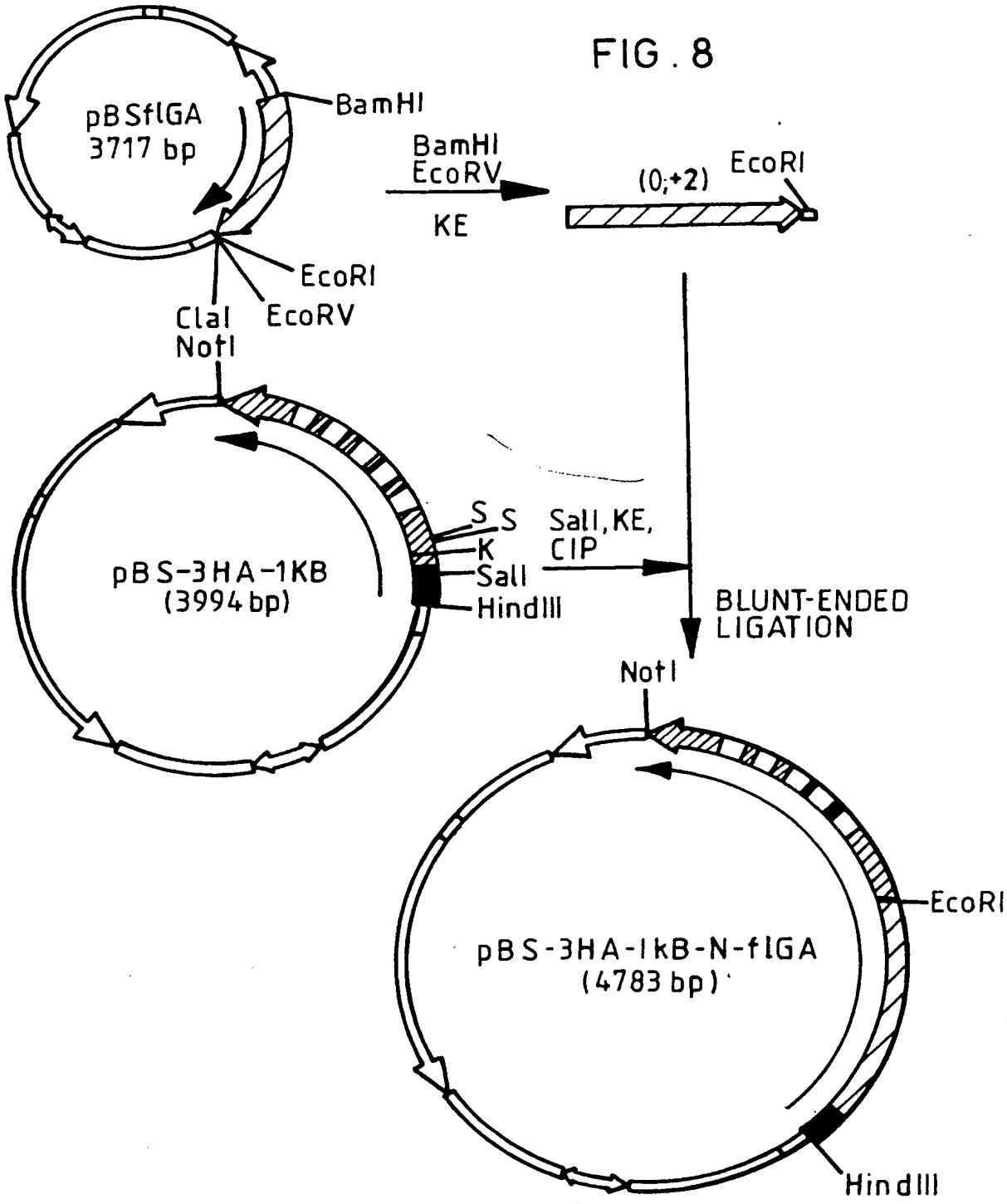
M G R D P P T
gc atg ggt cga **gat ccc ccg acc**
SphI/NcoI

|————————FLGA 244 aa————————>

G C R N_
ggc tgc agg aat tcg
EcoRI

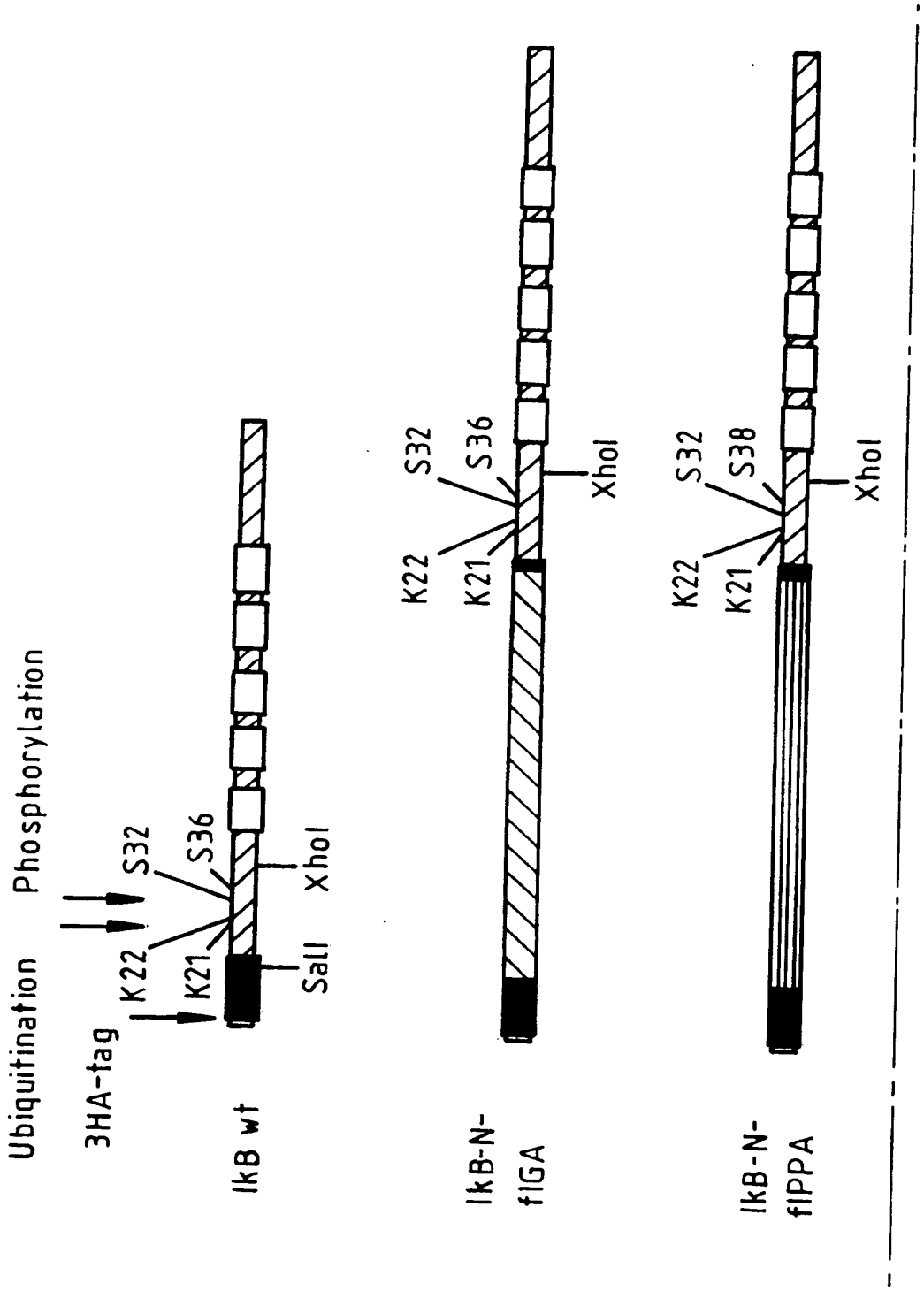
FIG. 7

FIG. 8



Cloning of pBS-3HA-IkB-N-flGA chimeric gene. KE indicates the treatment with Klenow enzyme, CIP means the dephosphorilations with calf intestine phosphatase. Scratched boxes indicate IkB sequences, filled boxes show hemagglutinin tag, diagonally scratched boxes show GA cassett. See the detailed explanation in the text

FIG. 9


Ubiquitination
Phosphorylation
3HA-tag
S32
S36
K22
K21
XhoI
SalI
IkB wt
IkB-N-fIGA
IkB-N-fIPPA
S38

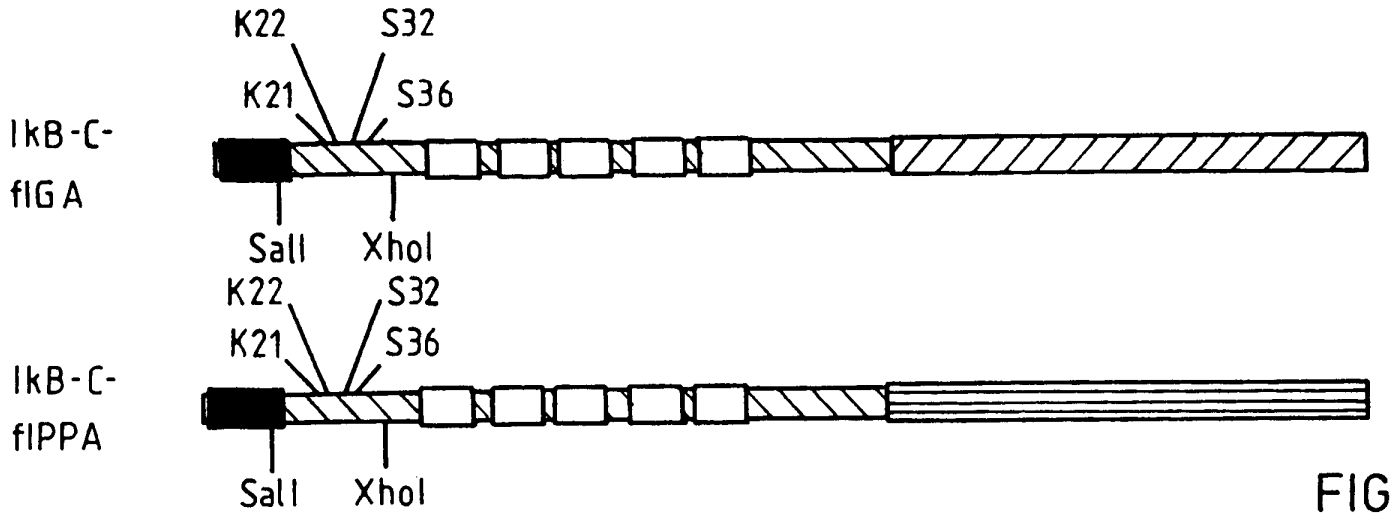
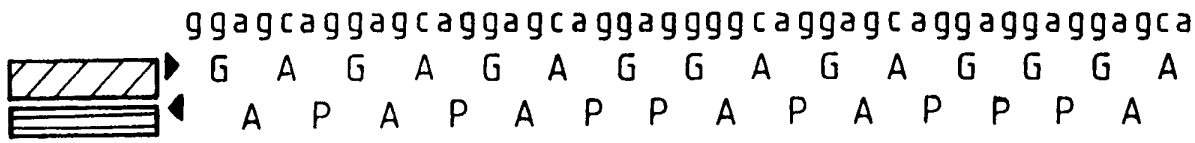


FIG. 9(contd.)



The maps of chimeric IkB genes. All the genes were cloned in pBS II KS(+). Stratched blocks indicate the wild type IkB sequence; ankyrin repeats are shown as open blocks. 3HA-tag marks three hemagglutinin epitopes on the 5' end of each gene. The positions of sygnal-dependent phosphorylation (serines in positions 32 and 36) are shown as S32 and S36, weil the sites of ubiquitination (lysines in positions 21 and 22) are marked as K21, K22. Diagonally stratched blocks correspond glycine-alanine repeat inserts; horizontally stratched ones indicate proline-alanine repeat.

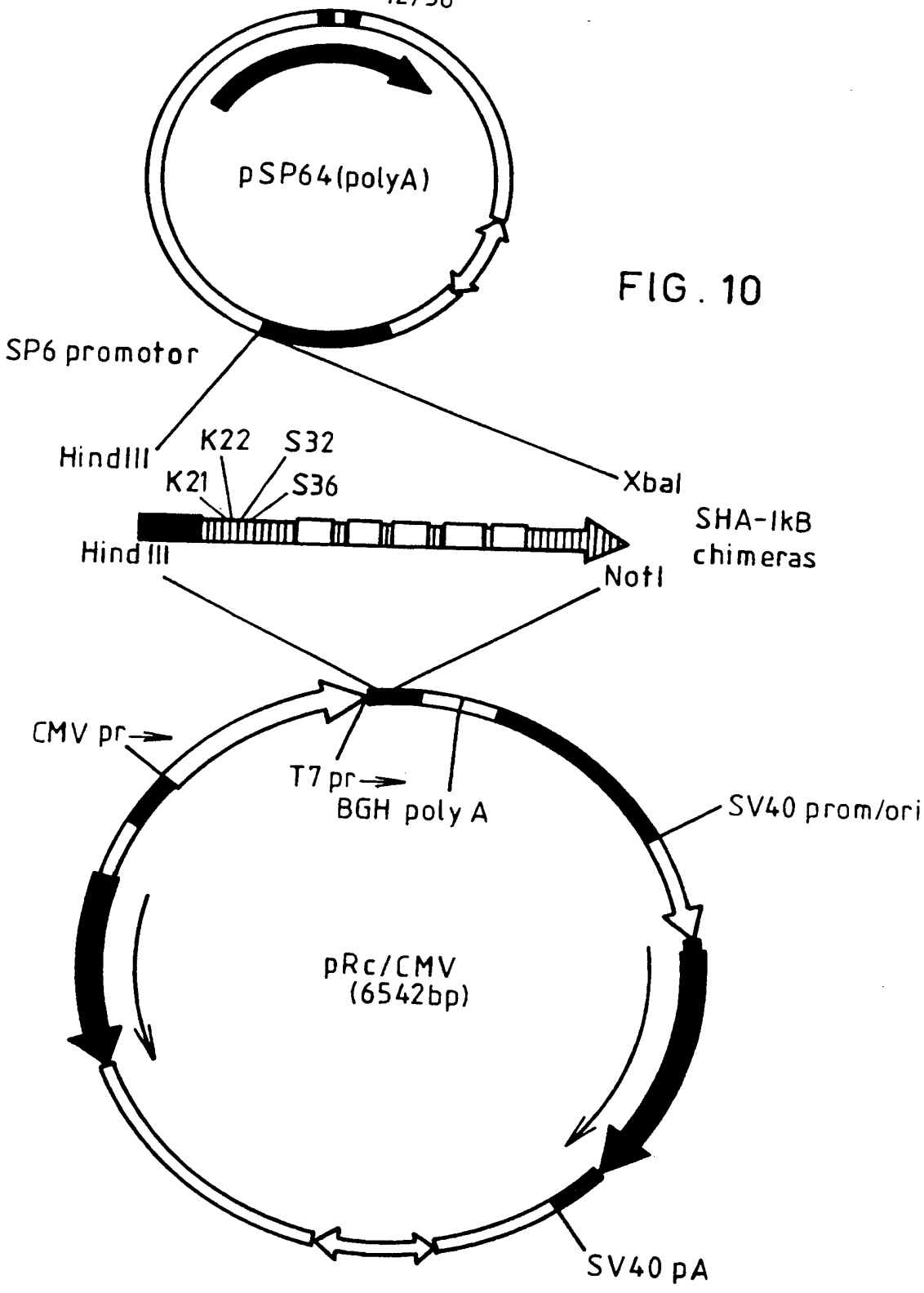


FIG. 10

The scheme of pSP64(polyA) and pRc/CMV series of constructs. The 3HA-IkB chimeras were cloned first in pRc/CMV vector in HindIII/NotI sites, then the inserts were recloned in pSP64(polyA)vector in HindIII/XbaI sites.

FIG. 11

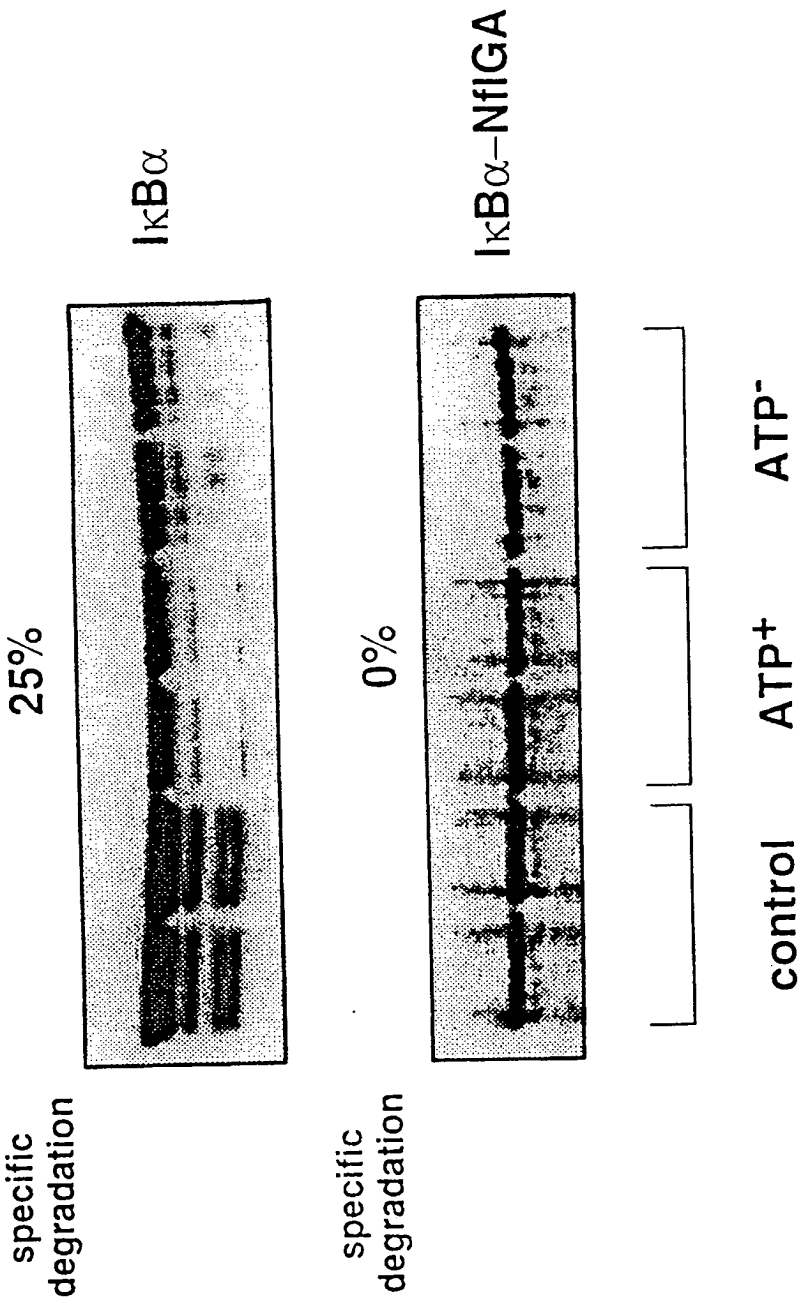

IκBα
IκBα–NfIGA
25%
0%
specific degradation
specific degradation
control
ATP+
ATP-

FIG. 12

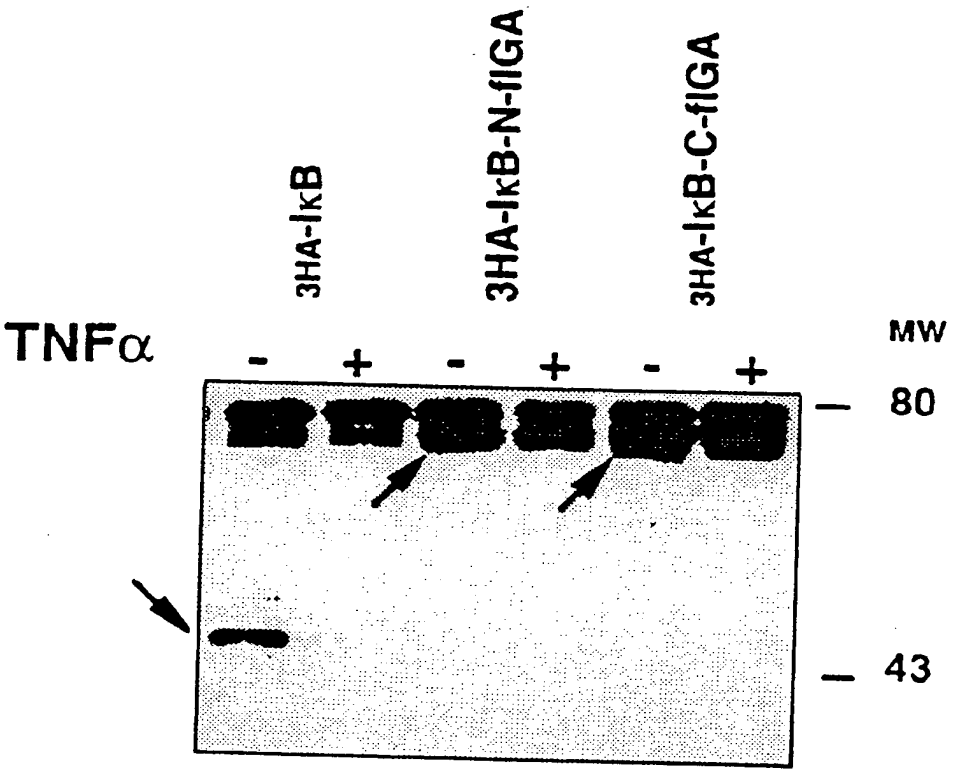


FIG. 13

MFQPAGHGQDWAMEGPRDGLKKERLVDDRHDSGLDSMKDEE

YEQMVKELREIRLQPQEAPLAAEPWKQQLTEDGDSFLHLAIIH

EEKPLTMEVIGQVKGDLAFLNFQNNLQQTPLHLAVITNQPGIA

EALLKAGGCDPELRDFRGNTPLHLACEQGCLASVAVLTQTCTPQ

HLHSVLQATNYNGHTCLHLASTHGYLAIVEHLVTLGADVNAQ

EPCNGRTALHLAVDLQNPDLVSLLLKCGADVNRVTYQGYSPY

QLTWGRPSTRIQQQLGQLTLENLQMLPESEDEESYDTESEFTE

DELPYDDCVFGGQRLTL

FIG. 14

GGCACGAGCC TCGCCAGCCA GCCGCCAGCA GCCTGCAGCC TGCACCCGCT CAGCCCCGCA

CAGCC ATG TTT CAG CCA GCT GGG CAC GGC CAG GAC TGG GCC ATG GAG GGC CCG CGG

GAT GGC CTC AAG AAG GAG CGC TTG GTG GAC GAT CGC CAC GAC AGC GGC CTG GAC TCC

ATG AAG GAC GAG GAG TAC GAG CAA ATG GTG AAG GAG CTG CGG GAG ATC CGC CTG CAG

CCG CAG GAG GCG CCG CTG GCC GCC GAG CCC TGG AAG CAG CAG CTC ACG GAG GAC GGA

GAC TCG TTC CTG CAC TTG GCA ATC ATC CAC GAA GAG AAG CCG CTG ACC ATG GAA GTC

ATT GGT CAG GTG AAG GGA GAC CTG GCC TTC CTC AAC TTC CAG AAC AAC CTG CAG CAG

ACT CCA CTC CAC TTG GCT GTG ATC ACC AAC CAG CCA GGA ATT GCT GAG GCA CTT CTG

AAA GCT GGC TGT GAT CCT GAG CTC CGA GAC TTT CGA GGA AAT ACC CCT CTA CAT CTT

GCC TGT GAG CAG GGC TGC CTG GCC AGT GTA GCA GTC TTG ACG CAG ACC TGC ACA CCC

CAG CAT CTC CAC TCC GTC CTG CAG GCC ACC AAC TAC AAT GGC CAC ACG TGT CTG CAC

CTA GCC TCT ACT CAC GGC TAC CTG GCC ATC GTG GAG CAC TTG GTG ACT TTG GGT GCT

GAT GTC AAC GCT CAG GAG CCC TGC AAT GGC CGG ACA GCC CTC CAC CTT GCG GTG GAC

CTG CAG AAT CCT GAC CTG GTT TCG CTC TTG TTG AAA TGT GGG GCT GAT GTC AAC AGG

GTA ACC TAC CAA GGC TAC TCC CCC TAC CAG CTT ACC TGG GGC CGC CCA AGT ACC CGG

ATA CAG CAG CAG CTG GGC CAG CTG ACC CTG GAA AAT CTC CAG ATG CTA CCC GAG AGC

GAG GAT GAG GAG AGC TAT GAC ACG GAG TCA GAA TTC ACA GAG GAT GAG CTG CCC TAT

GAT GAC TGT GTG TTT GGA GGC CAG CGT CTG ACA TTA TAA GTGGAAAGTG

GCAAAAAAGA ATGTGGACTT GTATATTTGT ACAAATAGAG TTTTATTTTT CTAAAAAAAA

AAAAAAAAAA A

FIG. 14 (contd.)

FIG. 15

MFQAAERPQEWAMEGPRDGLKKERLLDDRHDSGLDSMKDEE

YEMVKELQEIRLEPQEVPRGSEPWKQQLTEDGDSFLHLAIIHEE

KALTMEVIRQVKGDLAFLNFQNNLQQTPLHLAVITNQPEIAEA

LLGAGCDPELRDFRGNTPLHLACEQGCLASVGVLTQSCTTPHL

HSILKATNYNGHTCLHLASIHGYLGIVELLVSLGADVNAQEPC

NGRTALHLAVDLQNPDLVSLLLKCGADVNRVTYQGYSPYQLT

WGRPSTRIQQQLGQLTLENLQMLPESEDEESYDTESEFTEFTED

ELPYDDCVFGGQRLTL

FIG. 16

1 tgccgccgtc ccgcccgcca gcgccccagc gaggaagcag cgcgcagccc gcggcccagc

61 gcacccgcag cagcgcccgc agctcgtccg cgcc atg ttc caggcggccg agcgcccccа

121 ggagtgggcc atggagggcc cccgcgacgg gctgaagaag gagcggctac tggacgaccg

181 ccacgacagc ggcctggact ccatgaaaga cgaggagtac gagcagatgg tcaaggagct

241 gcaggagatc cgcctcgagc cgcaggaggt gccgcgcggc tcggagccct ggaagcagca

301 gctcaccgag gacggggact cgttcctgca cttggccatc atccatgaag aaaaggcact

361 gaccatggaa gtgatccgcc aggtgaaggg agacctggct ttcctcaact tccagaacaa

421 cctgcagcag actccactcc acttggctgt gatcaccaac cagccagaaa ttgctgaggc

481 acttctggga gctggctgtg atcctgagct ccgagacttt cgaggaaata ccccctaca

541 ccttgcctgt gagcagggct gcctggccag cgtgggagtc ctgactcagt cctgcaccac

601 cccgcacctc cactccatcc tgaaggctac caactacaat ggccacacgt gtctacactt

661 agcctctatc catggctacc tgggcatcgt ggagcttttg gtgtccttgg gtgctgatgt

721 caatgctcag gagccctgta atggccggac tgcccttcac ctcgcagtgg acctgcaaaa

781 tcctgacctg gtgtcactcc tgttgaagtg tggggctgat gtcaacagag ttacctacca

841 gggctattct ccctaccagc tcacctgggg ccgcccaagc acccggatac agcagcagct

901 gggccagctg acactagaaa accttcagat gctgccagag agtgaggatg aggagagcta

961 tgacacagag tcagagttca cggagttcac agaggacgag ctgccctatg atgactgtgt

1021 gtttggaggc cagcgtctga cgtta tga gt gcaaaggggc tgaaagaaca tggacttgta

1081 tatttgtaca aaaaaaaagt tttatttttc taaaaaaaga aaaaagaaga aaaaatttaa

1141 agggtgtact tatatccaca ctgcacactg cctagcccaa aacgtcttat tgtggtagga

1201 tcagccctca ttttgttgct tttgtgaact ttttgtaggg gacgagaaag atcattgaaa

1261 ttctgagaaa acttctttta aacctcacct ttgtggggtt tttggagaag gttatcaaaa

1321 atttcatgga aggaccacat tttatattta ttgtgcttcg agtgactgac cccagtggta

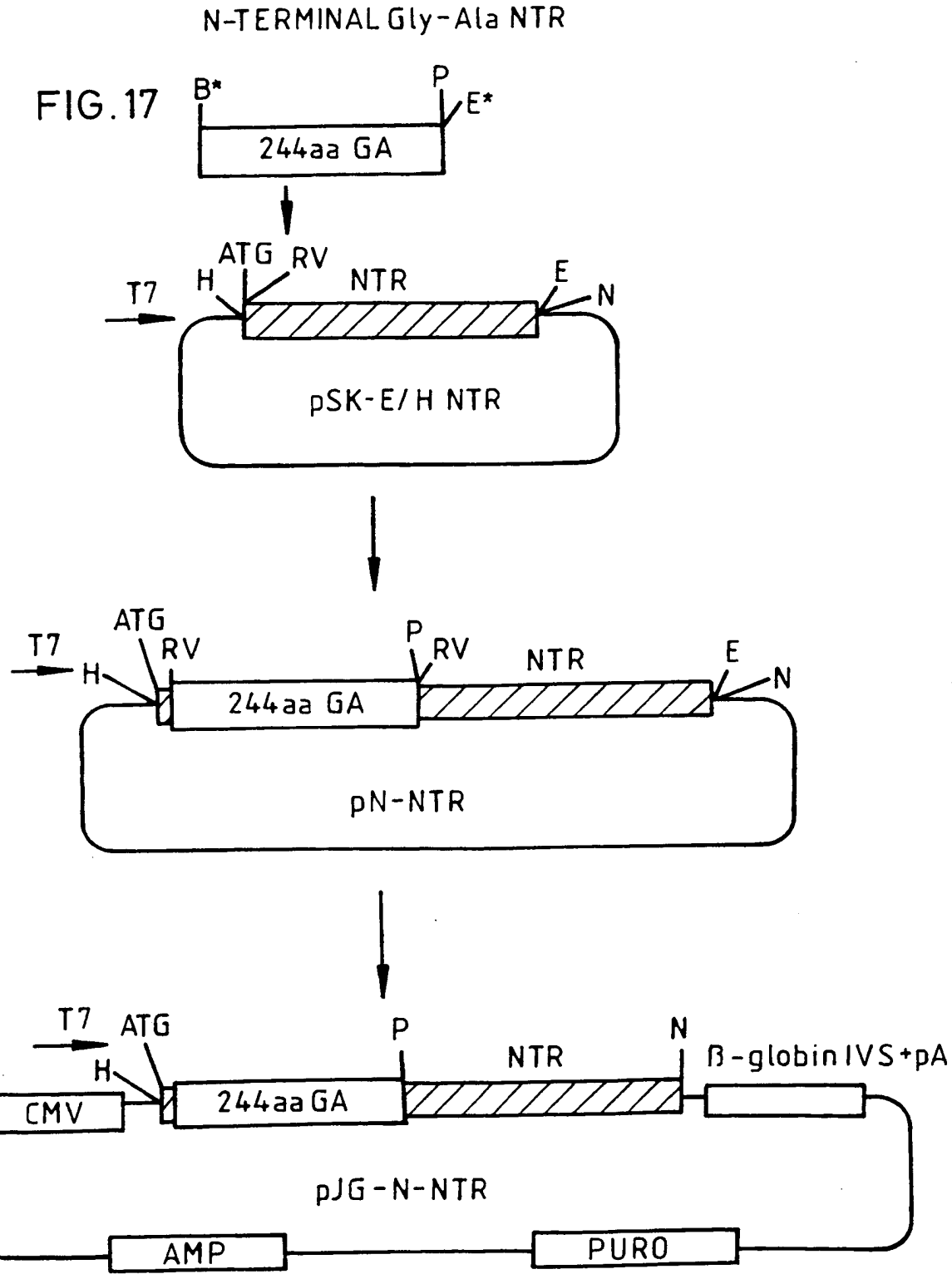
1381 tcctgtgaca tgtaacagcc aggagtgtta agcgttcagt gatgtggggt gaaaagttac

1441 tacctgtcaa ggtttgtgtt accctcctgt aaatggtgta cataatgtat tgttggtaat

1501 tattttggta cttttatgat gtatatttat taaagagatt tttacaaatg

FIG. 16(contd.)

FIG. 17


N-TERMINAL Gly-Ala NTR
B*
P
E*
244aa GA
ATG
RV
T7
H
NTR
E
N
pSK-E/H NTR
ATG
T7
RV
H
P
RV
NTR
E
N
244aa GA
pN-NTR
T7
ATG
P
N
H
NTR
ß-globin IVS+pA
CMV
244aa GA
pJG-N-NTR
AMP
PURO

22/36

C-TERMINAL Gly-Ala NTR

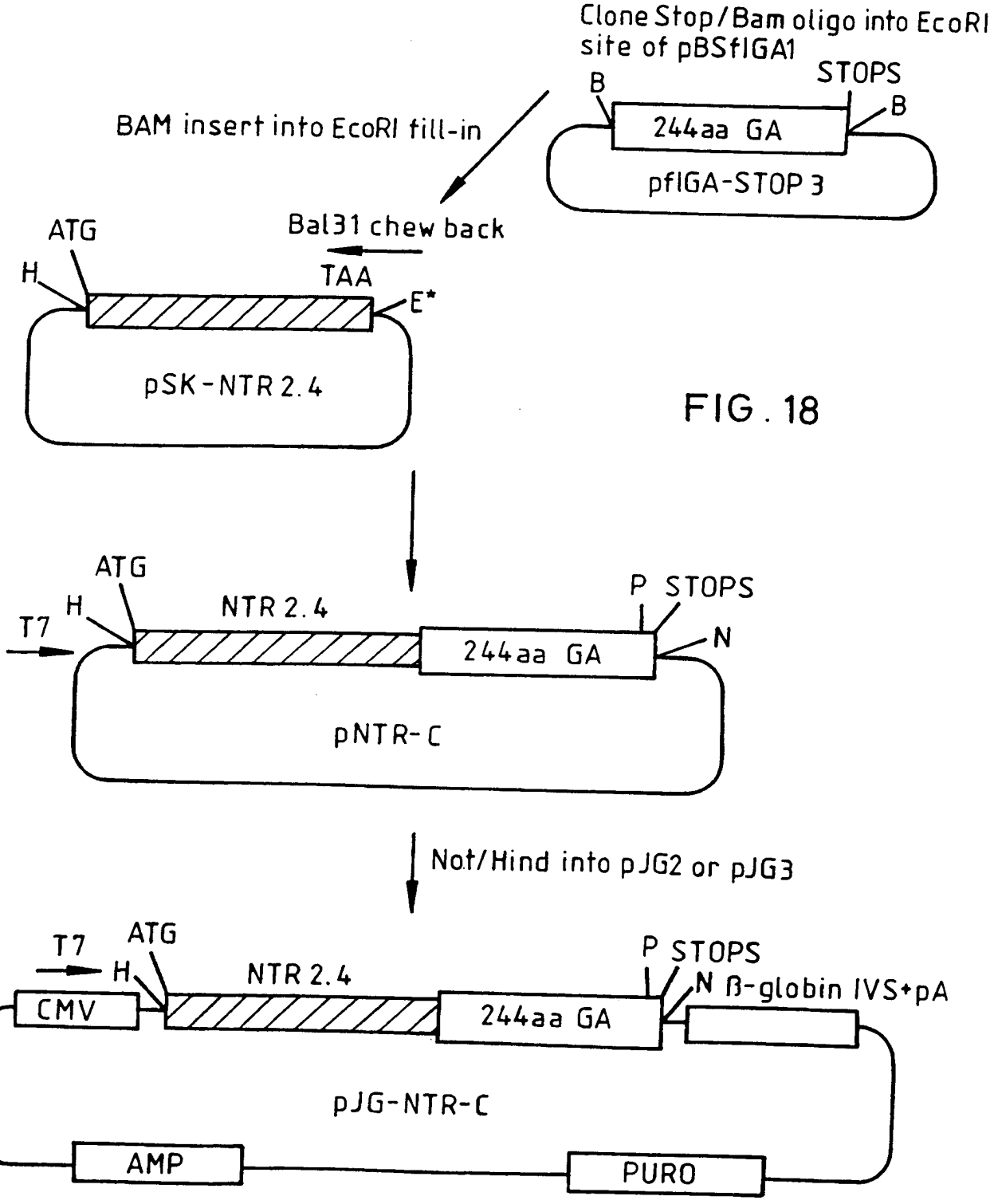


FIG. 18

FIG. 19

GLY-ALA OLIGO CONSTRUCTS

```
                                        1   2   3   4   5   6   7   8   9  10
SALI          CLAI  HINDIII     GLY ALA GLY ALA GLY GLY ALA GLY GLY ALA
GTCGACGGTATCGATAAGCTTGAT GGA GCT GGT GCT GGA GGT GCG GGT GGA GCT
 11  12  13  14  15  16  17  18  19  20  21  22  23  24  25
GLY ALA GLY GLY ALA GLY GLY ALA GLY ALA GLY GLY ALA GLY GLY
GGT GCT GGA GGT GCG GGT GGA GCT GGT GCT GGA GGT GCG GGT GGA G


ATCGAATTCCTGCAGCCCGGGGGATCCACT
     ECORI  PSTI    SMAI   BAMHI
```

```
                                        1   2   3   4   5   6   7   8   9  10
SALI          CLAI  HINDIII     GLY ALA GLY ALA GLY GLY ALA GLY GLY ALA
GTCGACGGTATCGATAAGCTTGAT GGA GCT GGT GCT GGA GGT GCG GGT GGA GCT
 11  12  13  14  15  16  17  18  19  20  21  22  23  24  25  26  27  28  29  30
GLY ALA GLY GLY ALA GLY GLY ALA GLY ALA GLY GLY ALA GLY GLY ALA GLY ALA GLY GLY
GGT GCT GGA GGT GCG GGT GGA GCT GGT GCT GGA GGT GCG GGT GGA GCT GGT GCT GGA GGT
 31  32  33
ALA GLY GLY
GCG GGT GGA G ATCGAATTCCTGCAGCCCGGGGGATCCACT
              ECORI  PSTI    SMAI   BAMHI
```

FIG. 20

PRO-ALA OLIGO CONSTRUCTS

```
                                        1   2   3   4   5   6   7   8   9  10
SALI          CLAI  HINDIII             PRO PRO ALA PRO PRO ALA PRO ALA PRO PRO
GTCGACGGTATCGATAAGCTTGA T CT CCA CCC GCA CCT CCA GCA CCA GCT CCA CCC
  11  12  13  14  15  16  17
ALA PRO PRO ALA PRO ALA PRO
GCA CCT CCA GCA CCA GCT CCA TCGAATTCCTGCAGCCCGGGGGATCCACT
                            ECORI PSTI  SMAI BAMHI
```

```
                                        1   2   3   4   5   6   7   8   9  10  11  12  13  14
 SALI          CLAI  HINDIII             PRO PRO ALA PRO PRO ALA PRO ALA PRO PRO ALA PRO PRO ALA
GTCGACGGTATCGATAAGCTTGA T CT CCA CCC GCA CCT CCA GCA CCA GCT CCA CCC GCA CCT CCA GCA
  15  16  17  18  19  20  21  22  23  24  25
PRO ALA PRO PRO ALA PRO PRO ALA PRO ALA PRO
CCA GCT CCA CCC GCA CCT CCA GCA CCA GCT CCA TCGAATTCCTGCAGCCCGGGGGATCCACT
                                          ECORI PSTI  SMAI BAMHI
```

FIG. 21(A)

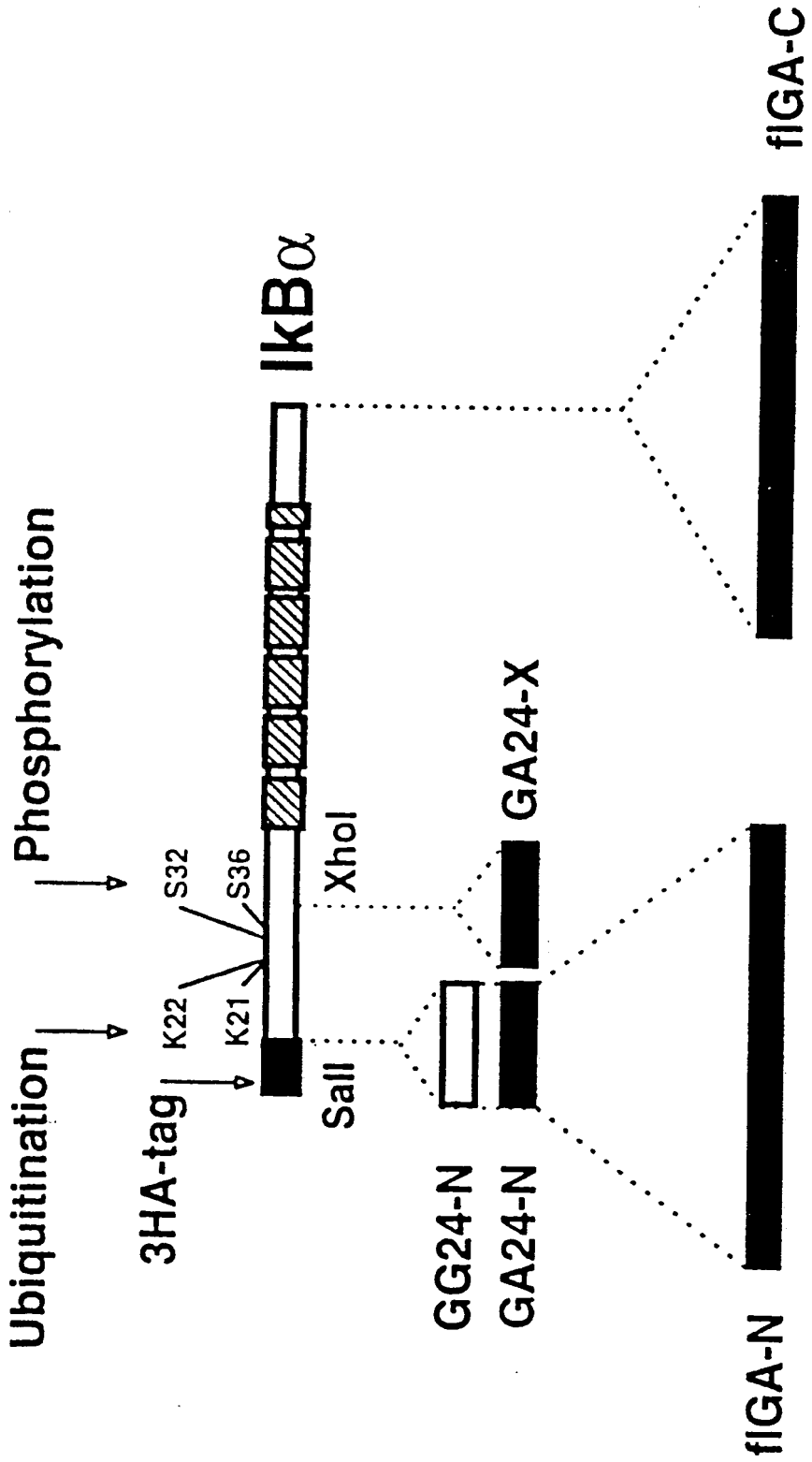

Phosphorylation
Ubiquitination
3HA-tag
S32
S36
K22
K21
IkBα
XhoI
SalI
GA24-X
GG24-N
GA24-N
fIGA-C
fIGA-N

FIG. 21(B)

flGA

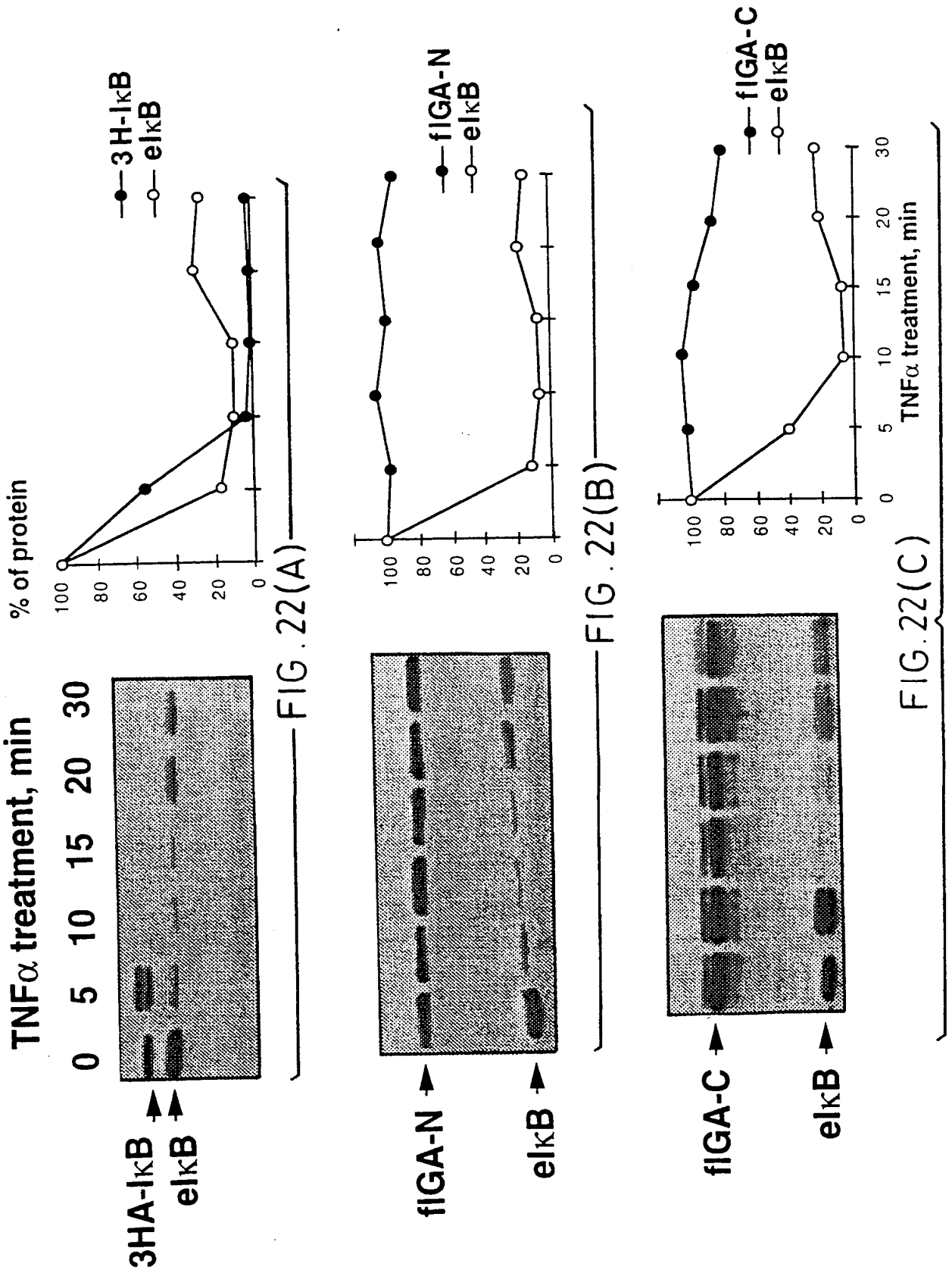
..*DPPTHGGT*GAGAGAGGAGAGGAGAGGG
AGAGGGAGGAGGAGGAGAGGGAGAGGGAG
GAGGAGAGGGAGAGGGAGGAGAGGGAGGA
GGAGAGGGAGAGGGAGGAGAGGGAGGAGG
AGAGGGAGAGGAGGAGGAGAGGAGAGGGA
GGAGGAGAGGAGAGGAGAGGAGAGGAGGA
GAGGAGGAGAGGAGGAGAGGGAGGAGAGG
GAGGAGAGGAGGAGAGGAGGAGAGGAGGA
GAGGGAGAGGAGAGGGG*RGRGGSGGRGCR*
NS..

GA24

..GGGAGAGGAGGAGGAGAGGAGAGA..

GG24

..GGGGGGGGGGGGGGGGGGGGGGGG..


TNFα treatment, min
0 5 10 15 20 30
3HA-IκB
elκB
% of protein
3H-IκB
elκB
100 80 60 40 20 0
FIG. 22(A)
fIGA-N
elκB
fIGA-N
elκB
100 80 60 40 20 0
FIG. 22(B)
fIGA-C
elκB
fIGA-C
elκB
0 5 10 15 20 30
TNFα treatment, min
100 80 60 40 20 0
FIG. 22(C)

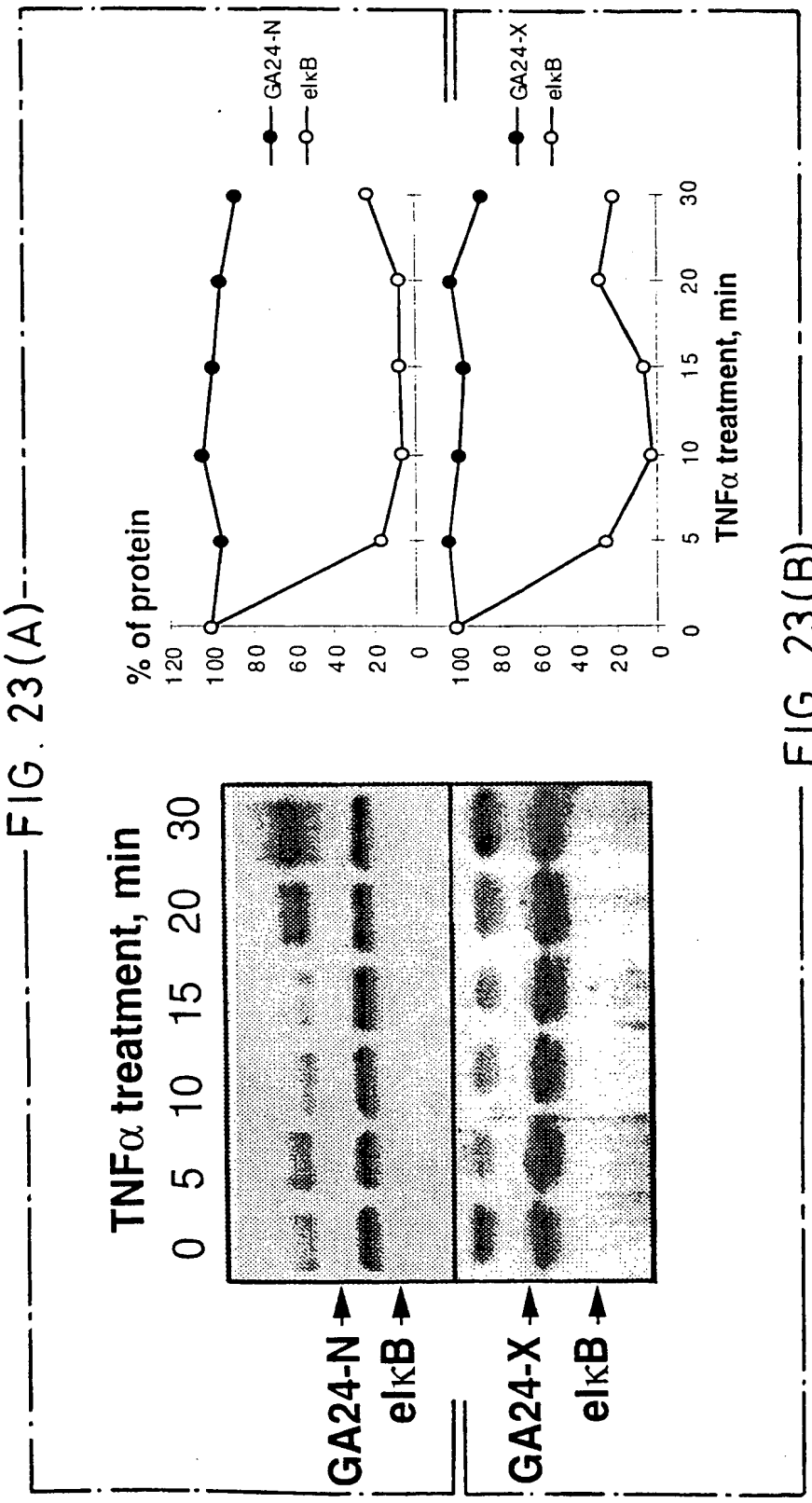


FIG. 23(A)

FIG. 23(B)

FIG. 24

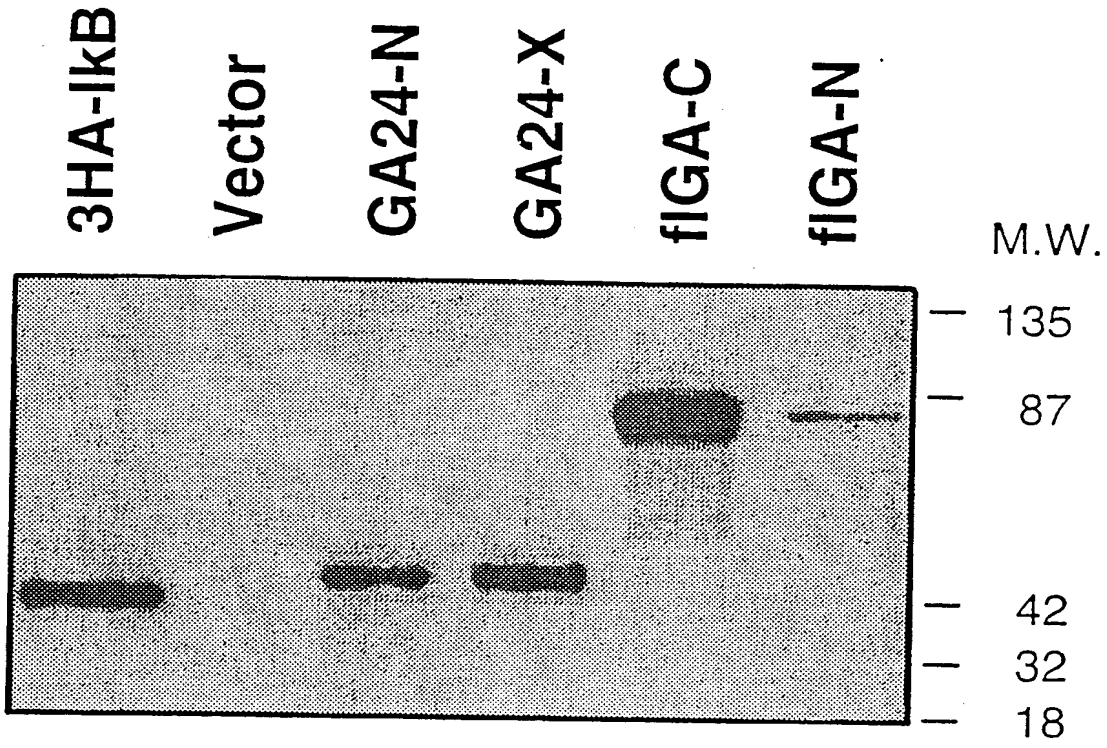
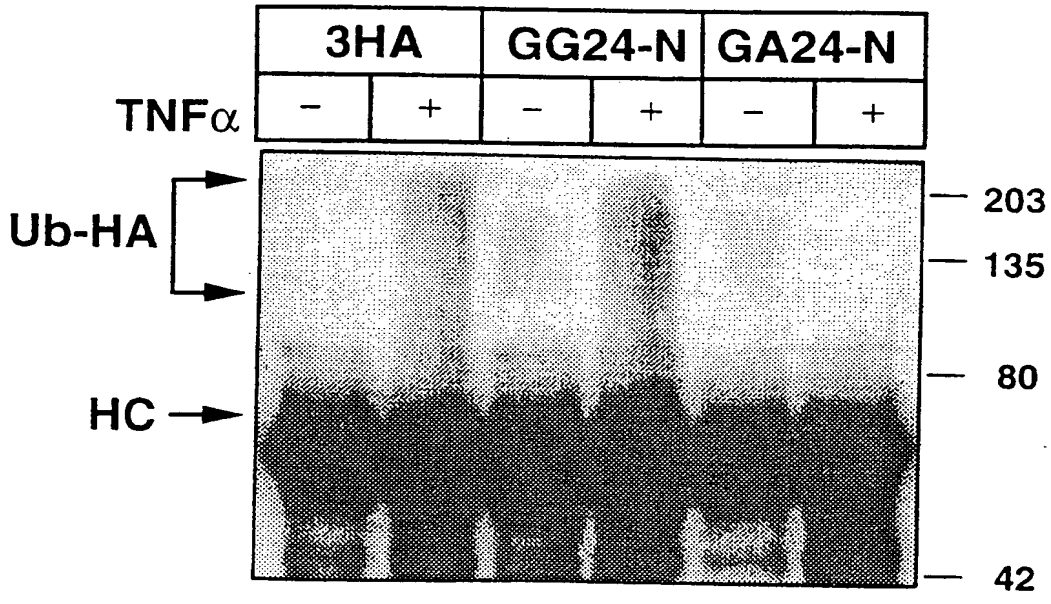

3HA-IkB
Vector
GA24-N
GA24-X
fIGA-C
fIGA-N
M.W.
135
87
42
32
18

FIG. 26


3HA
GG24-N
GA24-N
TNFα
−
+
−
+
−
+
Ub-HA
HC
203
135
80
42

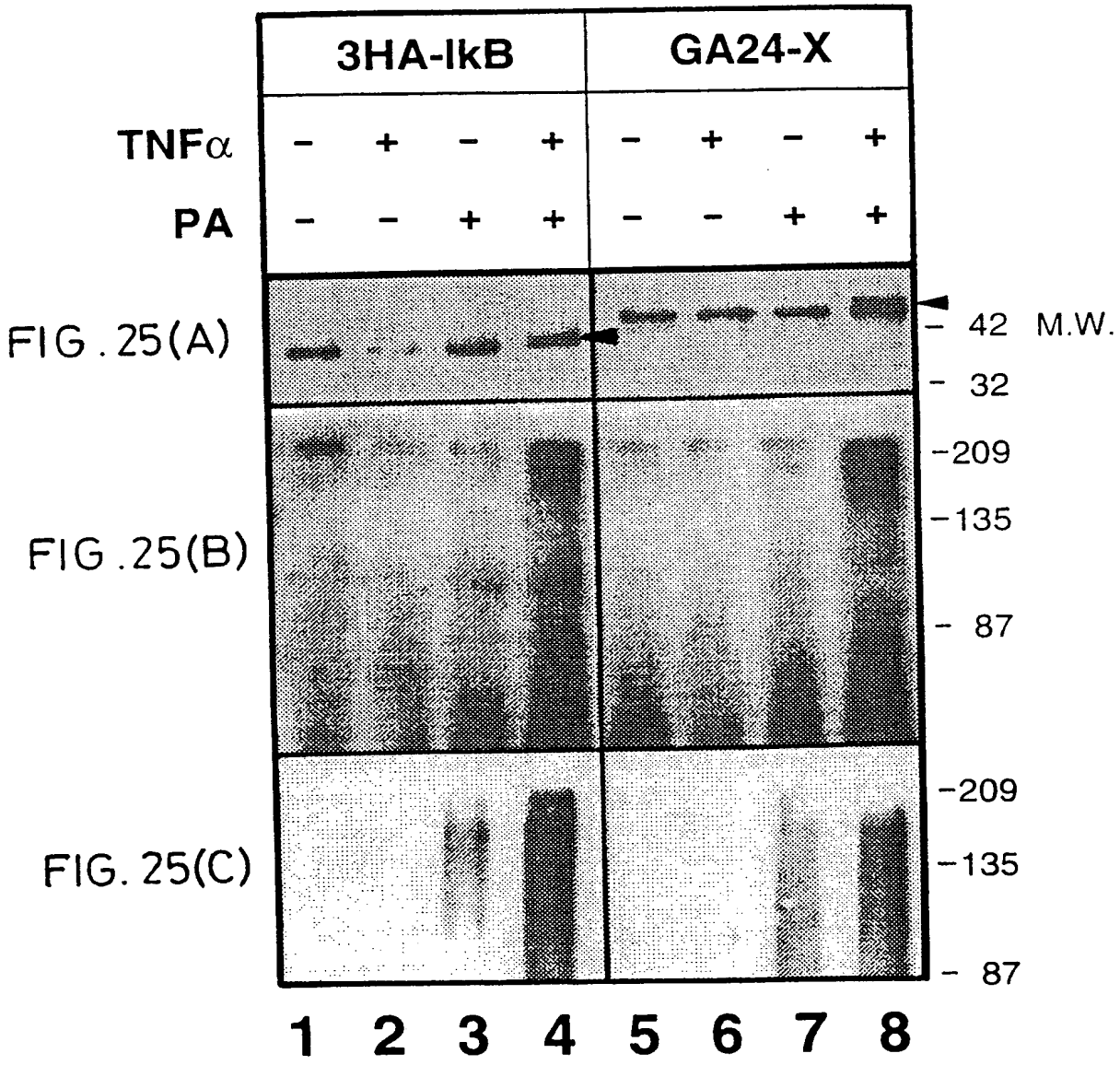


FIG. 25(A)

FIG. 25(B)

FIG. 25(C)

FIG. 27(A)

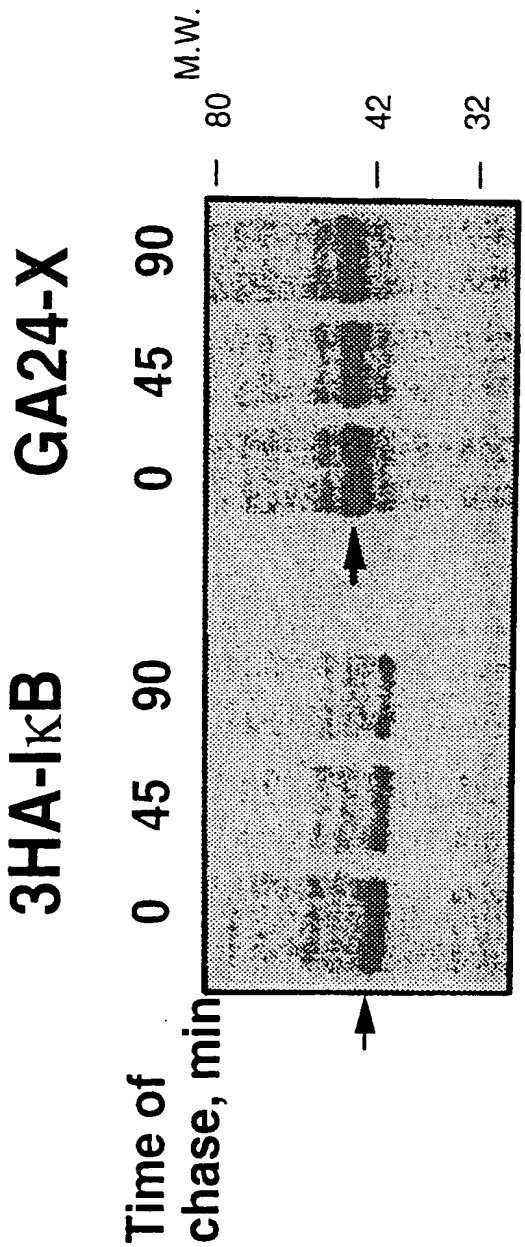


FIG. 27(B)

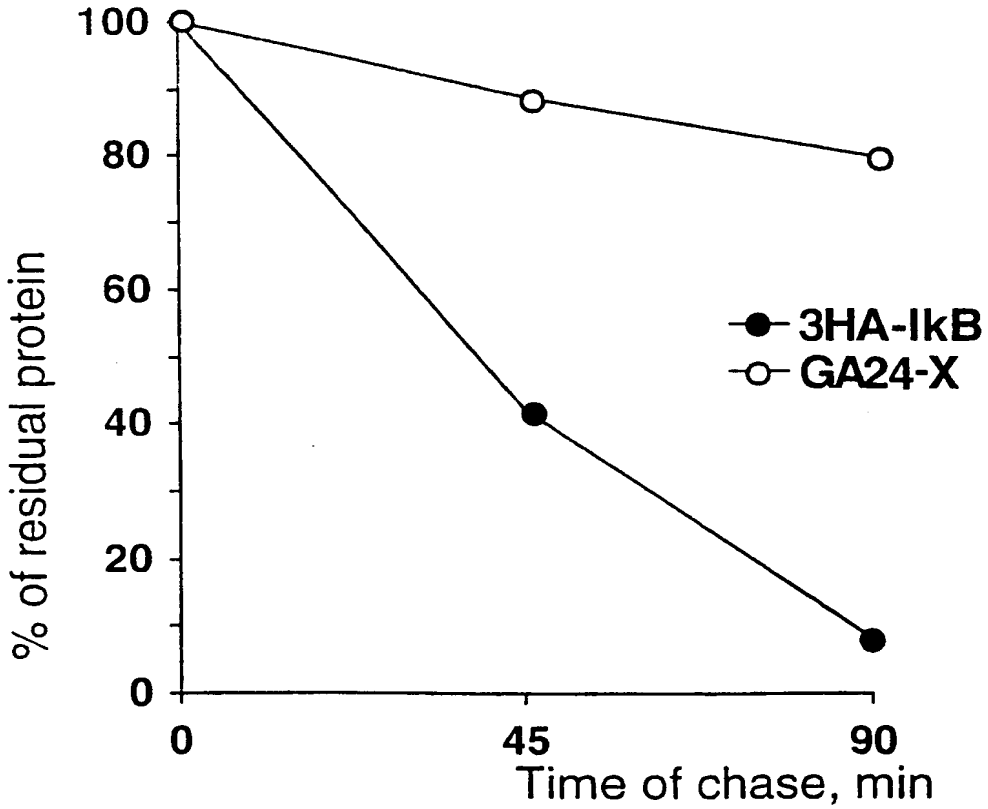

100
80
60
40
20
0
% of residual protein
0
45
90
Time of chase, min
3HA-IkB
GA24-X

FIG. 28(A)

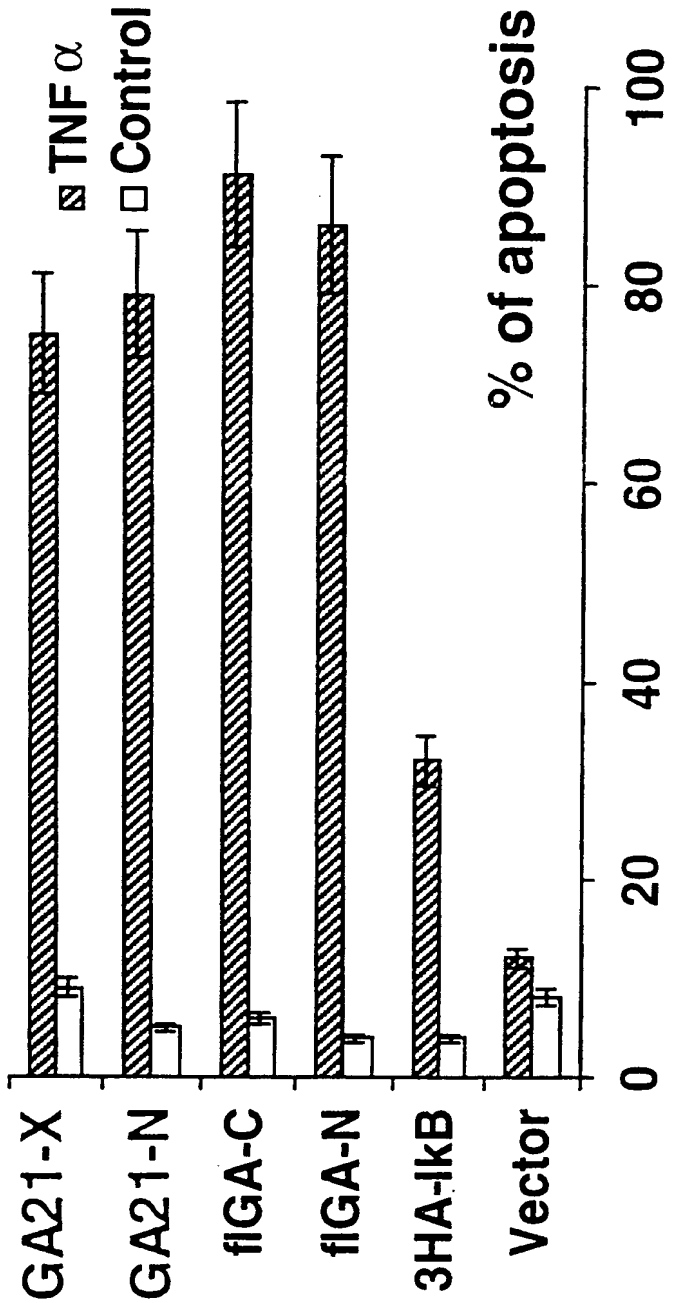

TNF α
Control
% of apoptosis
0
20
40
60
80
100
GA21-X
GA21-N
flGA-C
flGA-N
3HA-IkB
Vector

FIG. 28(B)

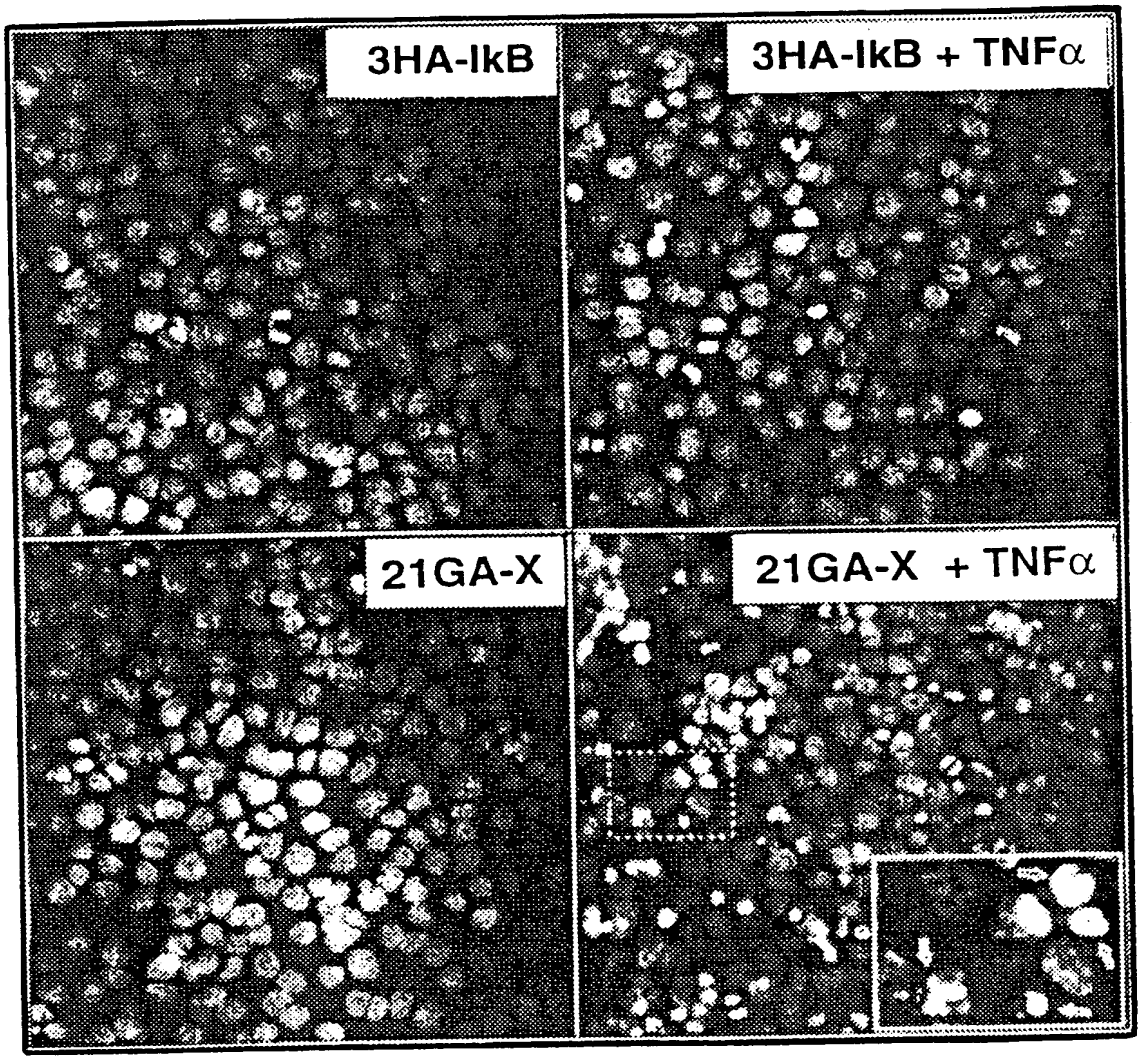


FIG. 29(A)

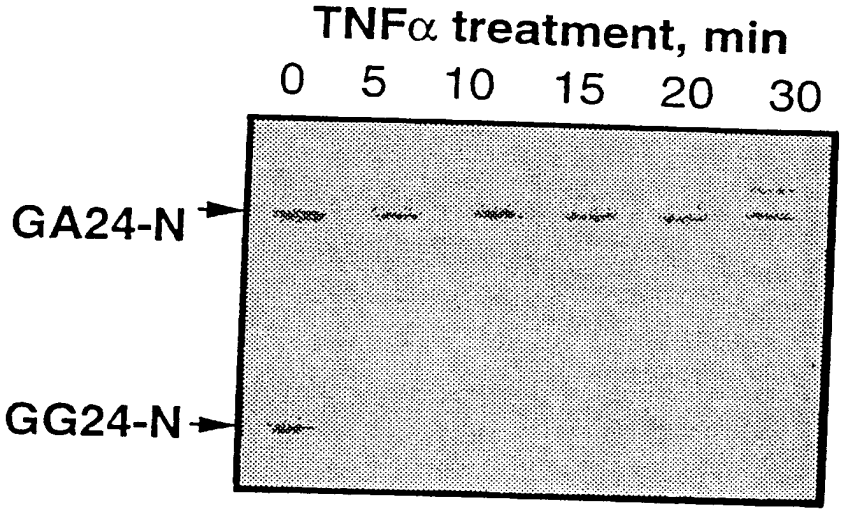


FIG. 29(B)

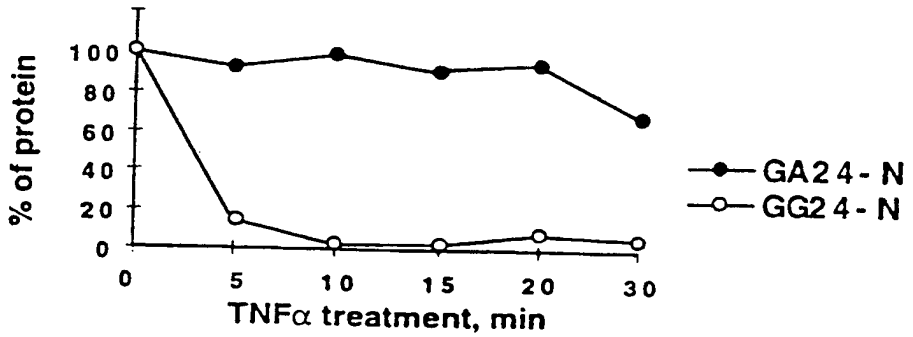


FIG. 30(A)

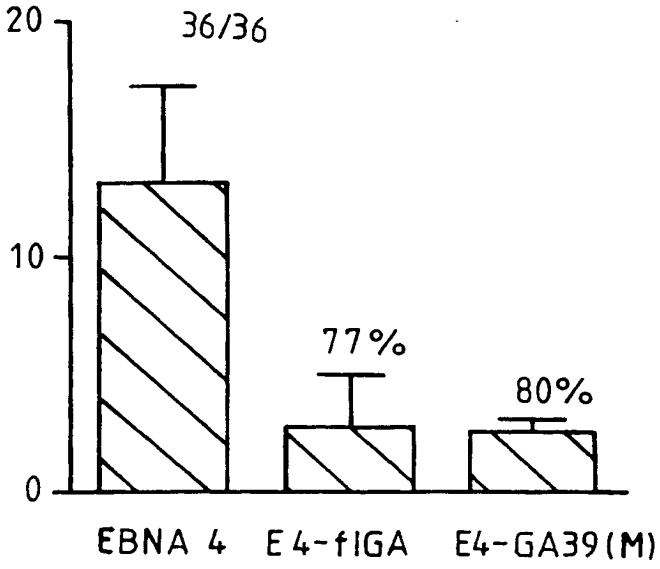


FIG. 30(B)

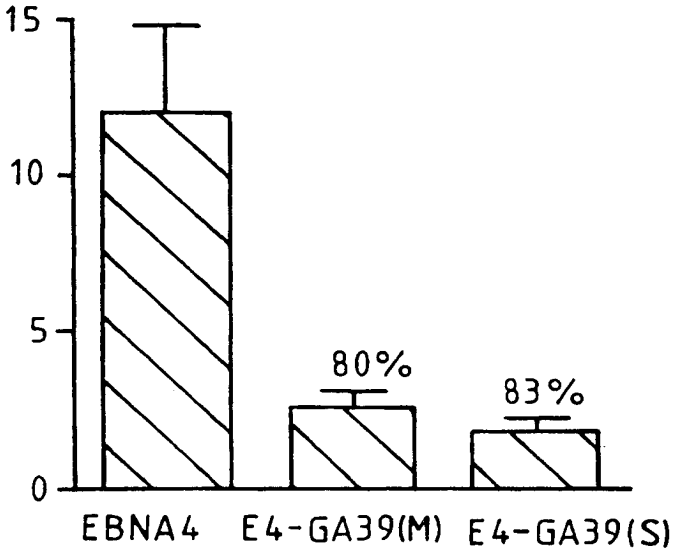
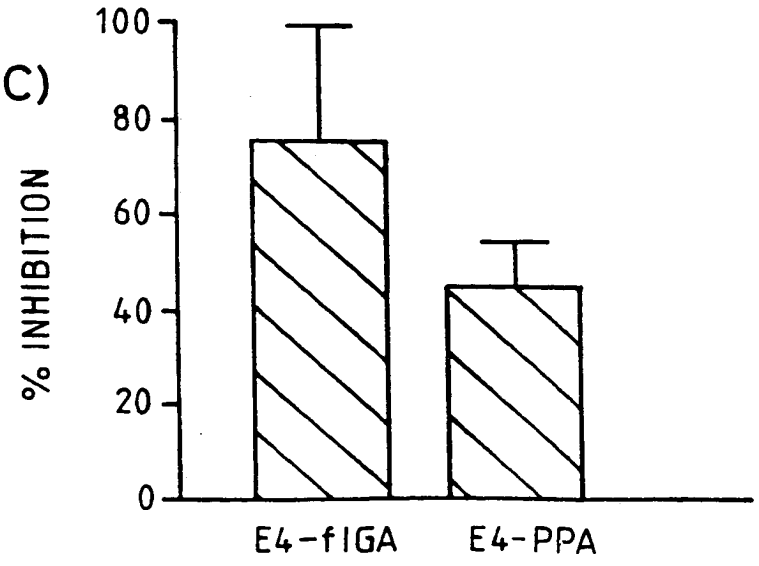


FIG. 30(C)



INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/IB 97/01508

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C12N15/10 C07K14/47 C07K14/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 32483 A (MASUCCI MARIA GRAZIA) 17 October 1996 whole document ---	1-30
X	LI LIN AND SANKAR GHOSH: "a glycine-Rich REgion in NF-kappaB p105 Functions as a Processing Signal for the Generation of the p50 Subunit" MOLECULAR AND CELLULAR BIOLOGY, vol. 16, no. 5, May 1996, pages 2248-2254, XP002062168 whole document, esp. Fig 1 --- -/--	1-30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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Date of the actual completion of the international search	Date of mailing of the international search report
15 April 1998	08. 05. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kronester-Frei, A

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INTERNATIONAL SEARCH REPORT

Interr. nal Application No
PCT/IB 97/01508

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LEVITSKAYA J. ET AL: "Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1" NATURE, vol. 375, 1995, pages 685-688, XP002062169 whole document, esp. Fig. 1	1-30
Y	US 5 122 448 A (VAUGHAN JOHN H ET AL) 16 June 1992 whole document, esp. column 2, line 40-column 8, line 55, claims	1-30
E	WO 97 46573 A (UNIV VIRGINIA) 11 December 1997 whole document	1-30
P,X	LEVITSKAYA J. ET AL.: "Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen-1" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 94, November 1997, pages 12616-12621, XP002062170 whole document, esp. page 12618, right column, Fig.6, Abstract	1-30
A	MANORAMA TEVARI ET AL.: "Rapid Induction in Regenerating Liver of RL/IF-1 and PHF, a novel kappaB Site-Binding Complex" MOLECULAR AND CELLULAR BIOLOGY, vol. 12, no. 6, June 1992, pages 2898-2908, XP002062171 whole text	18,19
A	WO 94 23045 A (BOEHRINGER INGELHEIM INTERNATIONAL) 13 October 1994 claims	1-30
A	EP 0 540 263 A (CANCER RES CAMPAIGN TECH) 5 May 1993 claims	26

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern nal Application No
PCT/IB 97/01508

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		AT 114671 T	15-12-1994
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		CA 1292839 A	03-12-1991
		DE 3587949 D	12-01-1995
		DE 3587949 T	06-07-1995
		DK 156786 A	07-04-1986
		EP 0191813 A	27-08-1986
		IE 65391 B	18-10-1995
		JP 6078359 B	05-10-1994
		JP 62500027 T	08-01-1987
		WO 8601210 A	27-02-1986
		US RE33897 E	21-04-1992
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		AU 6538094 A	24-10-1994
		CA 2159822 A	13-10-1994
		EP 0693129 A	24-01-1996
		JP 8508169 T	03-09-1996
EP 0540263 A	05-05-1993	AU 681337 B	28-08-1997
		AU 2776992 A	21-05-1993
		AU 3521597 A	19-02-1998
		EP 0638123 A	15-02-1995
		WO 9308288 A	29-04-1993
		JP 7501692 T	23-02-1995
		US 5633158 A	27-05-1997